MOLECULAR MECHANISMS OF AXON GUIDANCE

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Molecular Mechanisms of Axon Pathfinding

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Summary

Dorsolateral commissural neurons and sensory neurons of the dorsal root ganglion (DRG) are two of the frequently used neuronal subpopulations for axon guidance studies.

Commissural neurons extend their axons toward the midline, which they cross, before they turn rostrally. Although many of the guidance cues involved in the dorsoventral migration of commissural axons have been identified, very little is known about the cues that mediate the rostral turning of commissural axons and their subsequent growth in rostral direction. With a subtractive hybridization screen we have identified a number of candidate cues that we have functionally analyzed with *in ovo* RNAi. One of the candidate cues was found to be *SHH* that when downregulated lead to improper stalling of the commissural axons at the floor-plate exit point and erroneous caudal turns. Cyclopamine-mediated inhibition of Smoothened (Smo), the signaling component of the Shh receptor, failed to cause commissural axons to stall or turn caudally , suggesting that the Smoothened-Patched receptor complex was not involved in the function of Shh as a guidance cue for postcommissural axons. In contrast, downregulation of *HIP*, a recently identified receptor of Shh, reproduced the phenotypes observed after downregulation of *SHH*. Therefore, Shh's effect on postcommissural axons was mediated by Hip.

An interesting feature of DRG sensory neurons is the establishment of modality-specific central projections within the dorsal and the ventral horns of the spinal cord. For example, sensory neurons that innervate the skin have central projections that are confined to the dorsal horn (laminae I-III) while sensory afferents that supply muscle spindles project virtually unbranched through the medial half of the spinal cord and arborize in the ventral cord where they make direct synaptic contacts with motor neurons. This modality-dependent target specificity requires a set of subpopulation-specific guidance cues that direct the central processes of DRG neurons to their proper laminae within the spinal cord. A combination of limb ablation and exogenous neurotrophin delivery allowed for an enrichment of one subpopulation (nociceptive, mechanoreceptive and

proprioceptive) of sensory neurons compared to the others, since different subpopulations have a different dependency on neurotrophins for their survival. RNA pools from subpopulation- “enriched” DRGs were used as a starting material in a subtractive hybridization screen. With this screen we aim at the identification of candidate guidance cues that will be functionally analyzed in future experiments.

Zusammenfassung

Dorso-laterale Kommissuralneurone und sensorische Neurone der Spinalganglien sind zwei beliebte neuronale Modellpopulationen für axonale Wegfindungsstudien. Axone der Kommissuralneurone wachsen gegen die Mittellinie, überqueren diese und wachsen dann in rostraler Richtung weiter. Obwohl bereits viele Wegweisermoleküle identifiziert wurden, die die dorso-ventrale Navigation der Kommissuralaxone dirigieren, ist kaum etwas über jene bekannt, die für das anschliessende Wachstum in rostraler Richtung verantwortlich sind. In einem Screen, der auf subtraktiver Hybridisierung basierte, haben wir mehrere Kandidaten identifiziert und anschliessend auf deren Funktion als Wegweiser getestet. Dafür verwendeten wir eine neu entwickelte Technik - in ovo RNAi. Einer dieser Kandidaten wurde als SHH (Sonic Hedgehog) identifiziert. Der Verlust von SHH-Aktivität führte entweder zu fehlerhaftem Stillstehen von Kommissuralaxonen beim Verlassen der Bodenplatte, der Struktur, die die Mittellinie des Rückenmarks bildet, oder zur Drehungen der Axone in caudale Richtung. Da das Blockieren von Smoothened, der Signal übermittelnden Komponente des Shh-Rezeptors, mittels Cyclopamin keine Störungen der Kommissuralaxondrehung in die Längsachse verursachte, lag der Schluss nahe, dass der Rezeptorkomplex aus Patched und Smoothened nicht in die Shh-Funktion als Wegweiser für post-kommissurale Axone involviert war. Hingegen führte die Blockade von HIP (Hedgehog interacting protein), einem kürzlich identifizierten Rezeptormolekül für Shh, zu denselben Phänotypen wie der Verlust der SHH-Funktion. Dies deutete darauf hin, dass der Wegweisereffekt von Shh auf postkommissurale Axone von Hip vermittelt wurde.

Ein interessanter Aspekt in der Entwicklung der sensorischen Nervenzellen der Spinalganglien ist die modalitätsspezifische Verknüpfung der zentralen Ausläufer mit Zielzellen im dorsalen und ventralen Teil des Rückenmarks. Zum Beispiel bleiben die Ausläufer der sensorischen Nervenzellen, die die Haut innervieren, im dorsalen Horn (Laminae I-III) während sensorische Fasern, die die Muskelspindeln innervieren, durch die mediale Hälfte des Rückenmarks

projizieren. Erst im ventralen Horn verzweigen sie sich und bilden Synapsen mit Motoneuronen. Das Erreichen der modalitätsabhängigen Zielzellen erfordert eine Serie von populationsspezifischen Wegweisern, die die zentralen Ausläufer der sensorischen Nervenzellen zu ihren Zielgebieten im Rückenmark lenken.

Mit einer Kombination von Amputation der Beinanlage und Verabreichen von Neurotrophinen konnten wir Subpopulationen der sensorischen Spinalganglienneurone anreichern, denn nocicepitive, proprioceptive und mechanoreceptive Nervenzellen sind abhängig von verschiedenen Neurotrophinen für ihr Ueberleben. Aus diesen für Subpopulationen "angereicherten" Spinalganglien isolierten wir RNA, die als Ausgangsmaterial für einen Screen diente, der auf subtraktiver Hybridisierung basierte. Auf diese Weise hoffen wir Kandidaten zu isolieren, die als Wegweiser für sensorische Fasern dienen können. Die funktionelle Charakterisierung dieser Kandidatenmoleküle wird in zukünftigen Experimenten mittels der bereits entwickelten Methode – in ovo RNAi – erfolgen.

1. Introduction

The nervous system is the most complex organ of vertebrates. It processes, controls and mediates responses to a wealth of information that each organism receives as input from both the external but also the internal environment. In order for the nervous system to be functioning, a precise and complex spatial organization is required. Creating this organization during development involves solving two major problems: first, arranging the cells in the correct locations, and second, establishing the correct pattern of neuronal connections. However, the magnitude and complexity of the task involved in wiring the complex nervous system is daunting. In the vertebrate brain alone there are more than 10^{11} neurons, including hundreds of different cell types that have to be assembled into highly complex neuronal networks with over 10^{14} connections. It is clear that the ultimate challenge in developmental neurobiology is to understand how this intricate pattern of neuronal connections occurs with high precision during development. Uncovering to what extent this precision of connections reflects instructions derived from DNA, how these instructions leads eventually to meaningful patterns of guidance signals in the developing embryo and how these signals are interpreted by the growing axons have been some of the questions that neurobiologists have been trying to solve over the past decades.

1.1 Axon Migration *In Vivo*

Over a century ago, Ramón y Cajal made a landmark observation on the patterns of nerve process outgrowth and proposed that each elongating axon is lured to its target by diffusible molecules secreted from the target cell (**Fig 1**; Ramón y Cajal, 1892). For this reason the axons are equipped with a structure that Cajal original termed "*cono de crecimiento"* (growth cone). During development, the highly motile growth cone guides the elongating axon through the complex environment of developing tissues, senses and responds to all the guidance cues and when in contact with the target cell, the growth is remodelled

to form the synaptic connection. Although the ability of axons to migrate guided by the growth cone (also called “driving force”) and pathfinding has been recognized more than 100 years ago a fundamental question still remains: How does the growth cone read and accurately interpret the signals that it encounters during its journey within the developing environment.

Growth cones move forward, and thus axons elongate, mediated by cues present within their environment that direct them toward their appropriate targets. The correct interpretation of the environmental guidance cues leads to stopping, starting, turning and branching of the advancing axons in the correct locations; a process required for the proper wiring of the nervous system during development. A guidance cue must meet two requirements to direct a growth cone. First it should be inhomogeneously present in the environment, in some of the cases by forming a gradient, and it should also have a localized effect on the growth cone. Filipodia radiating out from the growth cone are well situated to sample environmental cues and to identify instructive and permissive substrata upon which additional growth can occur. The growth cone receives “signals” from the filopodia and translates them into alterations of the location and the rate of actin polymerization. Such alterations eventually lead to the response of the growth cone which can be turning, further growing, or even stalling.

To facilitate their navigation through developing tissue the axons break up their trajectories into short segments, each less than a few hundred micrometers long. By doing so they actually reduce the daunting task of reaching the target tissue to a series of simpler tasks of navigation. This certain approach has been demonstrated for the peripheral projections of sensory axons in insects (Ho and Goodman, 1982). The pathway they follow can be divided into discrete segments, each bounded by a specific cell or group of cells that marks the end of one segment and the beginning of the next. Specific ablation of these guidepost cells results in profound misrouting of the axons when they reach the vacant area (Tear et al., 1993). Further studies revealed the presence of guidepost cells that harbor important guidance information in a variety of species, including vertebrates.

An additional source of guidance information for developing axons comes from neighboring axons. The first pioneer axons that develop in an embryo navigate through an axon-free environment, but the majority of axons that follow project in an environment already crossed with a scaffold of earlier projecting axons. The role of the pioneer axons is actually to form the tract that will be followed from later developing axons and to facilitate their navigation at a time point when the environment has become complex. Therefore, axon guidance can be facilitated by a selective fasciculation of the developing axons with preexisting pioneer axons that have already set up the tract. Selective fasciculation requires a mechanism that allows specific recognition between axons of the same neuronal subpopulation while it excludes fasciculation with irrelevant axons present in the vicinity (Kolodkin et al., 1992; Harrelson and Goodman, 1988). However, guidance by selective fasciculation has been described only for invertebrates and there is no evidence that the same process exists in higher vertebrates (Stoeckli and Landmesser, 1995).

1.2 Four Guidance Forces

The realization that axonal trajectories are broken up to shorter segments, though revealing, does not provide information about the actual navigation of developing axons within the embryo. Embryological, tissue culture, and genetic experiments indicate that growth cones are guided by at least four different mechanisms: contact attraction, chemoattraction, contact repulsion and chemorepulsion (reviewed in Tessier-Lavigne and Goodman, 1996). Accumulating evidence suggests that these mechanisms act simultaneously and in a coordinated manner to orchestrate axon pathfinding and that they are mediated by evolutionary conserved ligand receptor systems (**Fig.1**).

Ramón y Cajal was the first to suggest that axon guidance might be controlled by long-range chemoattraction, a process similar to the chemotaxis of motile cells during which target cells secrete diffusible attractant substances that “lure” axons from a distance (Ramón y Cajal, 1892). In vitro experiments where neurons

cultured in the presence of their target cells turn towards these cells, demonstrate the existence of chemoattractants produced and secreted either from intermediate or final targets of the axons (Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988; Placzek et al., 1990; Heffner et al., 1990). Soon thereafter, long-range chemorepulsion was demonstrated with the finding that axons can be repelled by diffusible factors secreted from tissues that these axons avoid *in vivo* (Fitzgerald et al., 1993; Tamada et al., 1995; Colamarino and Tessier-Lavigne, 1995).

Apart from diffusible substances, axons can also be guided at short range by contact-mediated mechanisms regulated by non-diffusible, cell surface and extracellular matrix (ECM) molecules. In this case an adhesive and permissive physical substrate is required (Letourneau, 1975). Therefore, axon growth can be channeled by a corridor of permissive substrate bounded by repulsive signals (Keynes and Stern, 1984; Oakley and Tosney, 1993). In the same manner local repulsive cues can block the forward progression of axons (Wizenmann et al., 1993). The responses of developing axons to repulsive signals could vary from deflection and axonal arrest to more dramatic changes such as growth cone collapse and retraction (Fan and Raper, 1995; Kapfhammer and Raper, 1987).

In conclusion diffusible and non-diffusible substances secreted from target tissues, as well as more locally distributed cell-surface bound proteins could act simultaneously and mediate axon guidance. Thus, developing axons should express a certain subset of receptors on their membranes, in a time-specific manner, that would allow the proper sampling of the complex environment through which they have to navigate.

1.3 Neuronal Subpopulations for Axon Guidance Studies

Despite the complexity of the nervous system most of the studies within the field of developmental neurobiology have been performed with a few distinct populations of neurons. The spinal cord is an easily accessible structure of the developing nervous system and thus it represents one of the major areas of axon

growth and axon pathfinding research. Two different neuronal populations have been profoundly examined: the commissural axons, a subpopulation of interneurons that “transport” information originating in the periphery, to the brain for further analysis; and the sensory neurons of the dorsal root ganglion (DRG) that collect peripheral input and transport it to the spinal cord via central processes.

a. DRG sensory neurons

During development primary sensory neurons originate from progenitors that migrate from the neural crest and certain ectodermal placodes to the sites where sensory ganglia form (D` Amico-Martel and Noden, 1983). Gradually, two axonal processes grow in opposite directions from the cell bodies of these early neurons to reach their peripheral and central target fields (**Fig. 2**). As in other parts of the nervous system, the innervation of these target fields is associated with a period of neuronal death during which the superfluous neurons are eliminated, followed by a period of modification and refinement of connections.

The accessibility of the avian sensory ganglia from the earliest stages of development is the main reason that much of our understanding of the cellular and molecular basis of neuronal development is based on sensory neurons. The developing target fields of certain sensory ganglia are also well-defined and accessible for experimental studies, and this has allowed direct investigation of the regulatory influence of the target tissue on neuronal development.

An interesting feature of DRG sensory neurons is that they establish quite different central projections within the dorsal and the ventral horns of the spinal cord. For example, sensory neurons that innervate the skin have central projections that are confined to the dorsal horn (Brown et al., 1981). On the other hand, sensory afferents that supply muscle spindles (called Ia afferents) project virtually unbranched through the medial half of the spinal cord and arborize in the ventral cord where they make direct synaptic contacts with the motorneurons (Ozaki and Snider, 1997) (**Fig 2**). This selectivity occurs despite the fact that all

sensory neurons originate at the same time, reach the spinal cord via the same pathway, and that different types of afferents establish their central projections during the same period of time.

Apart from the central projections, also the peripheral projections of the sensory afferents have been extensively studied. In peripheral sensory nerves the earliest axons grow directly to their targets without branching or growing in aberrant directions. In lumbosacral nerves, for example, the cutaneous afferents from DRG grow to their target skin along a stereotyped set of pathways and establish their connections at a precise location (Scott, 1986). Sensory growth cones emerge after those of motorneurons, and, for the proximal part of their course their pathways are pioneered by motor axons. However, after ablation of motorneurons, sensory neurons retain the ability to map out a typical pattern of main nerve trunks and branches (Landmesser and Honig, 1986) and can establish normal cutaneous innervation patterns (Scott, 1988). It seems that sensory neurons, like motorneurons, are specified with respect to their targets and that specific guidance cues exist that influence the distal part of their course.

Furthermore, the homing behavior of displaced or misdirected axons (Ferguson, 1983) indicated that specific cues are widely distributed within the developing embryo and can be detected away from normal pathways suggesting the existence of soluble cues that act from a distance. Menesini-Chen et al., have shown that sensory growth cones can detect and respond by bidirectional movements to gradients of NGF and that gradients can be effective over a range *in vivo* (Menesini-Chen et al., 1978). To produce the highly stereotyped patterns of peripheral nerves, however, attractants would be expected to be regionally specific, to be available during early developmental stages, and to be produced at a level sufficient for free diffusion and the establishment of gradients. Apart from long-range guidance cues, short-range cues are also required in order to guide axons in a more precise manner along steps of their migration. Short-range guidance cues are usually membrane-bound proteins that create either a permissive or an inhibitory environment for the axons to grow. Since they do not

form gradients, they have a more precise and locally restricted effect on the pathfinding of axons.

Currently, the sensory afferents of the dorsal root ganglion attract a great deal of interest within the field of axon guidance as a great number of interesting issues still remain to be elucidated.

b. Commissural Neurons

In the developing CNS of a wide variety of bilaterally-symmetric organisms, many interneurons (neurons that connect with other neurons) project axons along trajectories that are either perpendicular or parallel to the midline. The vertebrate spinal cord represents a particularly good model system for the study of axon pathfinding in the vicinity of the midline. Commissural interneurons located at dorsolateral positions on either side of the spinal cord, extend axons that project along a circumferential pathway toward the floor plate, a small group of specialized ependymal cells that form the ventral midline of the spinal cord (Bovolenta and Dodd, 1990; Colamarino and Tessier-Lavinge, 1995). The earliest commissural projections travel along the lateral edges of the spinal cord until they reach the floor plate. The later projections extend along the same pathway but due to the development of the motorneurons in the ventral horns they are not in contact with the lateral border of the spinal cord for the most ventral part of their trajectory. Instead they follow a more direct, ventromedial route to the floor plate. After reaching the floor plate, commissural axons cross the midline (Bovolenta and Dodd, 1990). At the contralateral margin of the floor-plate most axons turn orthogonally and extend within the longitudinally-oriented ventral funiculus (**Fig 3**). Commissural axons apparently ignore guidance cues located in the ipsilateral side of the spinal cord and they subsequently respond to guidance cues present at the contralateral side of the spinal cord, since they are never observed to turn into the longitudinal axis prior to midline crossing.

Apart from commissural neurons, a second developing population of dorsolaterally positioned spinal neurons (known as associational neurons), also

extend axons along a transverse path toward the midline. However, these axons perform a right angle turn and extend parallel to floor-plate prior to reaching the floor plate (Colamarino and Tessier-Lavigne, 1995).

Several studies performed in Drosophila, chicken embryos and mice have already identified a great number of both diffusible and contact-dependent guidance cues that can guide commissural axons towards the floor plate and mediate midline crossing (reviewed in Stoeckli, 1998). However, the mechanism and the guidance cues responsible for the right angle turn of commissural axons after midline crossing remain largely unknown, and are an area of active research.

1.4 Ligands and Receptors Implicated in Axon Guidance

A large number of the guidance cues and their cell surface receptors that orchestrate the wiring of the nervous system, have now been identified (Huber et al., 2003). Further studies have revealed a mechanistic conservation among guidance molecules, short-range and long-range, attractive and repulsive. Since both guidance molecules and mechanisms of axon guidance have been conserved during evolution, insights gained in invertebrates can be relevant to vertebrates and vice versa.

a. Netrins

The netrins consist of a small family of phylogenetically conserved and secreted proteins with aminoacid sequence similarity to proteins of the laminin family (Hedgecock et al., 1990; Serafini et al., 1994). Extensive characterization of netrin function in *C.elegans*, *Drosophila* and vertebrates has revealed the ability of netrins to function as both neuronal chemoattractants and repellents (reviewed in Chisholm and Tessier-Lavigne, 1999) An example of netrin bifunctionality is observed in vertebrates, where netrin-1 functions *in vitro* and *in vivo* to attract spinal cord commissural axons to the floor plate and *in vitro* to repel trochlear

motor axons (Colamarino and Tessier-Lavigne, 1995; Serafini et al., 1996). These opposing guidance responses to netrins may depend in part upon the activation of different receptor complexes within each neuronal subpopulation.
The receptors of netrins have been found to be Deleted in Colorectal Cancer (DCC) and its orthologs, proteins with large extracellular domains composed of multiple immunoglobulin (Ig) repeats, fibronectin type III (FNIII) repeats, and large cytoplasmic domains with three conserved motifs (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). Moreover, it was shown that ligand-mediated multimerization of the DCC proteins is required for netrin-induced events (Stein and Tessier-Lavigne, 2001). On the other hand, studies of UNC-6 (orthologue of vertebrate netrin-1) in *C.elegans* revealed that netrin's repulsive effect requires an additional receptor, UNC-5, together with the DCC ortholog UNC-40. Furthermore, these studies suggest that different regions of UNC-6 are responsible for functional associations with these two receptors (Lim and Wadsworth, 2002). In other words, the effect of netrin-1 on a given axon depends on the receptors present on its membrane at any given time.

b. Slits

Members of the Slit family of axon guidance molecules control a wide range of physiological processes during neural development including axon pathfinding (Kidd et al., 1999; Plump et al., 2002), axonal and dendritic branching (Whitford et al., 2002) and neuronal cell migration (Kramer et al., 2001; Wu et al., 2001). Slit proteins are structurally conserved among species and contain several protein motifs (Rothberg et al., 1990). They have been most extensively characterized with respect to their role in orchestrating axonal navigation at the midline. The *Drosophila* Slit protein is strongly expressed in specialized CNS midline glial cells and provides a repulsive cue that prevents ipsilaterally projecting neurons from crossing the midline and contralaterally projecting neurons from recrossing the midline (Rajagopalan et al., 2000; Simpson et al., 2000). In vertebrates three different slits have been identified to date with

overlapping but distinct patterns of expression throughout development (Marillat et al., 2002). Insights from Slit mutant mice revealed that Slit-1 and Slit-2 function as repellents for retinal ganglion axons, providing a “surround” repulsion that directs these axons along an appropriate path both before and after crossing the optic chiasm. Similar results were obtained from various other CNS tracts including the corpus callosum, thalamocortical projections and the lateral olfactory tract (Bagri et al., 2002; Nguyen-Ba-Charvet et al., 2002).

Studies in *Drosophila*, *C.elegans* and mammals identify the Robo family of transmembrane proteins as the receptors of Slits (Brose et al., 1999; Kidd et al., 1998; Zallen et al., 1998). Robo proteins are phylogenetically conserved Ig super family members with large extracellular domains composed of Ig and FNIII repeats and large cytoplasmic domains lacking catalytically active domains. The intracellular domains are composed of CC motifs and they are required for Slit-mediated chemorepulsion as well as for silencing the netrin-mediated chemoattraction (Bashaw and Goodman, 1999; Stein and Tessier-Lavigne, 2001).

c. Semaphorins

The Semaphorins belong to yet another large family of phylogenetically conserved, secreted and membrane-associated proteins, members of which are capable of mediating both repulsive and attractive axon guidance events during neural development (reviewed in Raper et al., 2000). Recently, the spectrum of biological functions attributed to semaphorins has been extended considerably to include roles in immune system function and development of the cardiovascular system (Trodjman et al., 2002). The family contains more than 30 members that are defined by a conserved ~ 500 aminoacid extracellular semaphorin domain and can be classified in eight subfamilies depending on their structural similarities and species of origin.

Although the conserved transmembrane proteins plexins are essential signal transducing components of most semaphorins (reviewed in Tamagnone and

Comoglio, 2000), other proteins that bind to semaphorins have been identified. Neuropilin-1 and 2 (Npn-1 and Npn-2) act as coreceptors for the secreted class 3 semaphorins. The Neuropilins are transmembrane proteins with short cytoplasmic tails that facilitate the binding of the class 3 semaphorins to plexin A receptors (Nakamura et al., 1998)

d. Ephrins

Eph tyrosine kinases, receptors for ephrins, are encoded by the largest family of receptor tyrosine kinase (RTK) genes in the mammalian genome. These receptors are divided in two subclasses, EphA receptors (EphA1-EphA8), which bind the GPI-linked ephrinAs (ephrinA1-ephrinA5), and EphB receptors (EphB1-EphB6), which bind to transmembrane ephrin-Bs (ephrinB1-ephrinB3; reviewed in Cutforth and Harrison, 2002). In addition to their prominent roles as regulators of axon guidance, ephrins and Ephs control many other biological functions including cell migration, vascular development, tissue border formation and synaptic plasticity (Knoll and Drescher, 2002). Because ephrins are membrane attached, interactions between ephrins and Ephs require intercellular contact. It is remarkable that ephrin/Eph complexes transduce signals bidirectionally into both receptor (Eph)-expressing cells and ligand (ephrin)-expressing cells in what is known as “forward” and “reverse” signaling, respectively (reviewed in Kullander and Klein, 2002). There is ample evidence to indicate that both forward and reverse mode of signaling of ephrin-Eph are critical for axon guidance during neural development (Cooke and Moens, 2002; Holmberg and Frisen, 2002; Knoll and Drescher, 2002).

e. Receptor Protein Tyrosine Phosphatases

Members of the large family of protein tyrosine phosphatases (PTPs) have been implicated in a wide range of physiological processes including cell adhesion, cell migration, and development of the immune and nervous systems. Genetic

analysis in *Drosophila* has implicated several RPTPs in the control of axon fasciculation, defasciculation (Desai et al., 1997) and axon guidance of motor axons (Krueger et al., 1996). Moreover, mammalian RPTPκ, RPTPδ and RPTPμ (Burden-Gulley et al., 2002; Drosopoulos et al., 1999; Wang and Bixby, 1999) as well as chick PTPσ (Rashid-Doubell et al., 2002; Stepanek et al., 2005) have been implicated in the growth and guidance of several populations of developing vertebrate neurons.

f. Extracellular Matrix Proteins and their Receptors

Many ECM molecules, including the laminin, tenascin, collagen, and thrombospondin families, as well as fibronectin, vitronectin and a variety of proteoglycans, can act either as promoters or inhibitors of neurite outgrowth and extension *in vitro* (Bixby and Harris, 1991; Hynes and Lander, 1992). Receptors for ECM molecules are predominantly integrins, Ig superfamily members and proteoglycans. On the basis of their *in vitro* and *in vivo* activities and *in vivo* expression patterns, many ECM molecules are expected to play roles in axon guidance, but little is known about actual guidance functions *in vivo.* In *Drosophila*, loss of laminin A function results in the stalling of a subset of sensory axons while interfering with integrin function in *Xenopus* retinal axons *in vivo* causes a foreshortening of the axons (Garcia-Alonso, et al., 1996; Lilienbaum et al., 1995).

g. Cell Adhesion Molecules (CAMs) as Ligands and Receptors

Two large families of CAMs function during axon pathfinding: the immunoglobulin (Ig) and cadherin superfamilies (reviewed in Rutishauer, 1993). Many members of these two families can mediate homophilic adhesion, functioning as both a ligand on one cell and a receptor on another (Cunningham et al., 1987). Some members can also function as heterophilic ligands or receptors for distinct cell surface or ECM molecules (Felsenfeld et al., 1994). How many neural CAMs are

encoded in any one genome is still unknown, although there are at least 10 in *Drosophila* and more than 50 in mammals. Although some Ig CAMs contain cytoplasmic regions with protein tyrosine kinase activity or protein phosphatase domains (Bellosta et al., 1995), most do not, despite their apparent roles as signaling receptors (Kunz et al., 1996).

These molecules have been profoundly studied and their role in axon navigation has been demonstrated with either in vivo and/or in vitro experiments. Although the identification of these major guidance cues has increased our understanding of how the nervous system is wired, many guidance events observed during development do not appear to be accounted for by any of the already identified guidance cues. Moreover, the number of the known guidance cues and receptors seems rather small relative to the vast complexity of the nervous system wiring. Thus, additional guidance cues and receptors probably remain to be discovered.

1.5 Morphogens as Guidance Cues

Over the last few years members from three other families of secreted signaling molecules have been demonstrated to act as guidance cues: the Wingless (Wnt), the Hedhehogs (Hh) and the Bone Morphogenetic Proteins (BMPs). These findings came initially as a surprise as these molecules have been previously identified as morphogens controlling cell fate and tissue patterning. The common characteristic of all these secreted proteins is that they provide graded positional information, which can be interpreted by responding cells as either a cell fate specification signal or one for axonal pathfinding.

a. The Hedgehog Family

Hedgehogs can be found in insects and vertebrates. There is a single hedgehog gene in flies, and three in mammals: sonic hedgehog (Shh), indian hedgehog (Ihh) and desert hedgehog (Dhh). Shh is secreted by the notochord and by floor-

plate cells at the ventral midline of the neural tube, and functions as a graded signal for the generation of distinct classes of neurons along the dorsoventral (DV) axis of the neural tube (reviewed by Jessell, 2000; Ingham and McMahon, 2001; Marti and Bovolenta, 2002).

Apart from its morphogenetic function responsible for the patterning of the neural tube, Shh has been also shown to act as a chemoattractant for commissural axons in addition to netrin-1 (Charron et al., 2003). The attractive effect of Shh on commissural axons is a direct one and is not mediated via a re-patterning of the neural tube as at the developmental stage, at which the experiments were performed, the spinal cord has lost the competence to be re-patterned by Shh (Charron et al., 2003). Moreover, it was demonstrated that Shh utilizes the same signaling pathway in commissural axons as the one responsible for the function of Shh as a morphogen. Patched and Smoothened form a receptor complex that in the presence of Shh leads to the activation of the members of the Gli transcription factor family that leads eventually to alterations in gene expression (Ingham and McMahon, 2001)

In addition to its role in commissural axon navigation, Shh has been also shown to play a role in the axon growth of retinal ganglion (RGC) cell axons. Trousse et al., provided evidence that Shh expression at the chiasm border helps define a barrier within the ventral midline that serves to guide RGC axons, and suggest that Shh may be acting on the axons directly, rather than indirectly via a repatterning of the chiasm (Trousse et al., 2001).

The opposite effects of Shh on commissural and retinal axons (attraction and repulsion) might be due to an intrinsic or extrinsic factor that modulates cyclic nucleotide levels, in the same way that extrinsic factors can convert Netrin attraction to repulsion via the modulation of the cyclic nucleotide levels (Hopker et al., 1999). It is also possible that these two effects are mediated by distinct signaling pathways that result in opposite guidance effects – a possibility that has precedent in the case of netrins which can attract axons by activating DCC family receptors, and repel them by activating UNC5 family receptors (Tessier-Lavigne and Goodman, 1996; Dickson, 2002).

b. The BMP family

The roof plate of the neural tube is the major source of inductive signals responsible for the generation of dorsal cell types (Lee and Jessell, 1999). The expression of several members of the Bone Morphogenic Proteins (BMPs) is upregulated at the time point when dorsal neurons are generated, and it has been shown that some of them are necessary for the normal specification of dorsal neurons (Lee et al., 2000).

Studies of the *Ntn1* and *Dcc* mutants revealed that the commissural axons are able to follow their normal trajectory for approximately the first third of their trajectory before becoming misrouted, even in the absence of netrin's attractive effect (Serafini et al., 1996; Fazeli et al., 1997). This finding suggested the presence of additional guidance cues that repel commissural axons during the initial stage of their migration. Studies that followed demonstrated that BMP6 and BMP7, two BMP family members expressed by the roof plate, have the ability to repel commissural axons in the developing spinal cord (Ausburger et al., 1999; Butler and Dodd, 2003)

c. The Wnt family

Roof plate cells, apart from BMPs, produce and secrete several members of the Wnt family (reviewed by Lee and Jessell, 1999). Although Wnt-1 and Wnt-3A are required for the specification of dorsal neurons (Muroyama et al., 2002), it remains still an open question of whether they have a morphogenic function in this system.

The fact that Wnt proteins are able to reorganize the cytoskeleton during axonal growth and growth cone extension (Hall et al., 2000) suggested that the same proteins might also be involved in axon guidance. Soon after, studies in the CNS of *Drosophila* revealed that Wnt-5 can repel commissural axons from the posterior commissure (Yoshikawa et al., 2003). Following studies showed that

Derailed (Drl), a receptor tyrosine kinase, mediates the repulsive effect of Wnt-5 on commissural axons. Indeed loss of Wnt-5 function results in pathfinding defects similar to those seen in the Drl mutants, and overexpression of Wnt-5 throughout the midline blocked the formation of the anterior commissure (Yoshikawa et a., 2003). These results suggested that Wnt-5 acts as a chemorepellent on the commissural axons and that this response is mediated by Drl.

Furthermore, Lyuksyutova et al., provided evidence suggesting that the activity responsible for the rostral guidance of post-crossing commissural axons is an increasing posterior to anterior gradient of a diffusible attractant (Lyuksyutova et al., 2003). Subsequently, it was shown that several members of the Wnt family could influence the growth of commissural axons after crossing the midline. Biochemical and genetic data identified Wnt-4 as a chemoattractant for post-crossing commissural axons that forms a posterior-anterior gradient along the midline (Luyksyutova et al., 2003).

1.6 *In ovo* RNAi

The development of high-throughput approaches has altered the way genes, including those encoding guidance cues, are analyzed. Genome-sequencing projects as well as large-scale screens provide a tremendous amount of information about the genetic make up of an organism. Unfortunately, the long lists of genes expressed in specific tissues or distinct phases of an organism's life provide little or no information about the function of the expressed proteins.

In order to increase the pace of functional gene analysis new model systems are required for large-scale reverse genetic analyses and functional screens. Additionally, in the field of developmental neurobiology, the model systems should also be easily accessible and efficient in producing functional read-outs. So far these criteria were met only by invertebrate systems; however, for many questions vertebrate systems are required. Up to now the mouse was the animal model of choice, as a great arsenal of genetic approaches has been well

established. Unfortunately, the mouse suffers several disadvantages such as high costs for maintenance, the time required producing genetically modified mice and the difficult in accessibility during development.

A model organism that satisfies most, if not all, of these requirements is the chicken embryo that has been widely used in developmental biology but the lack of genetic techniques established for this animal model restricted its use. However, studies in our lab showed that the combination of RNAi (Fire et al., 1998) and *in ovo* electroporation (Itasaki et al., 1999) is a very efficient and specific tool for gene silencing in chicken embryos (Pekarik et al., 2003; Stoeckli, 2003; Bourikas and Stoeckli, 2003). We have shown that the down-regulation of three IgCAMs (namely *AXONIN1*, *NGCAM* and *NRCAM*) with *in ovo* RNAi was sufficient to reproduce the specific pathfinding errors of commissural axons observed after blockage of the same proteins with function blocking antibodies (Pekarik et al., 2003). Moreover, further studies showed specific downregulation both at the mRNA and the protein level (Bourikas and Stoeckli, 2003; Bourikas et al., 2005). This novel method of specific gene silencing eliminates the disadvantages of the chicken embryo as a model system and its application in a temporarily and spatially controlled manner allows researchers to distinguish the dual function of proteins during development such as morphogens that mediate tissue patterning at early developmental stages but also axon guidance later on in development.

1.7 Aim of my Thesis

The aims of my PhD thesis were:

A. The advancement of the *in ovo* RNAi technique

B. The characterization of Shh as a guidance cue for the commissural axons

C. The identification of candidate guidance cues responsible for the proper targeting of the central processes of DRG sensory axons in different laminae of the spinal cord

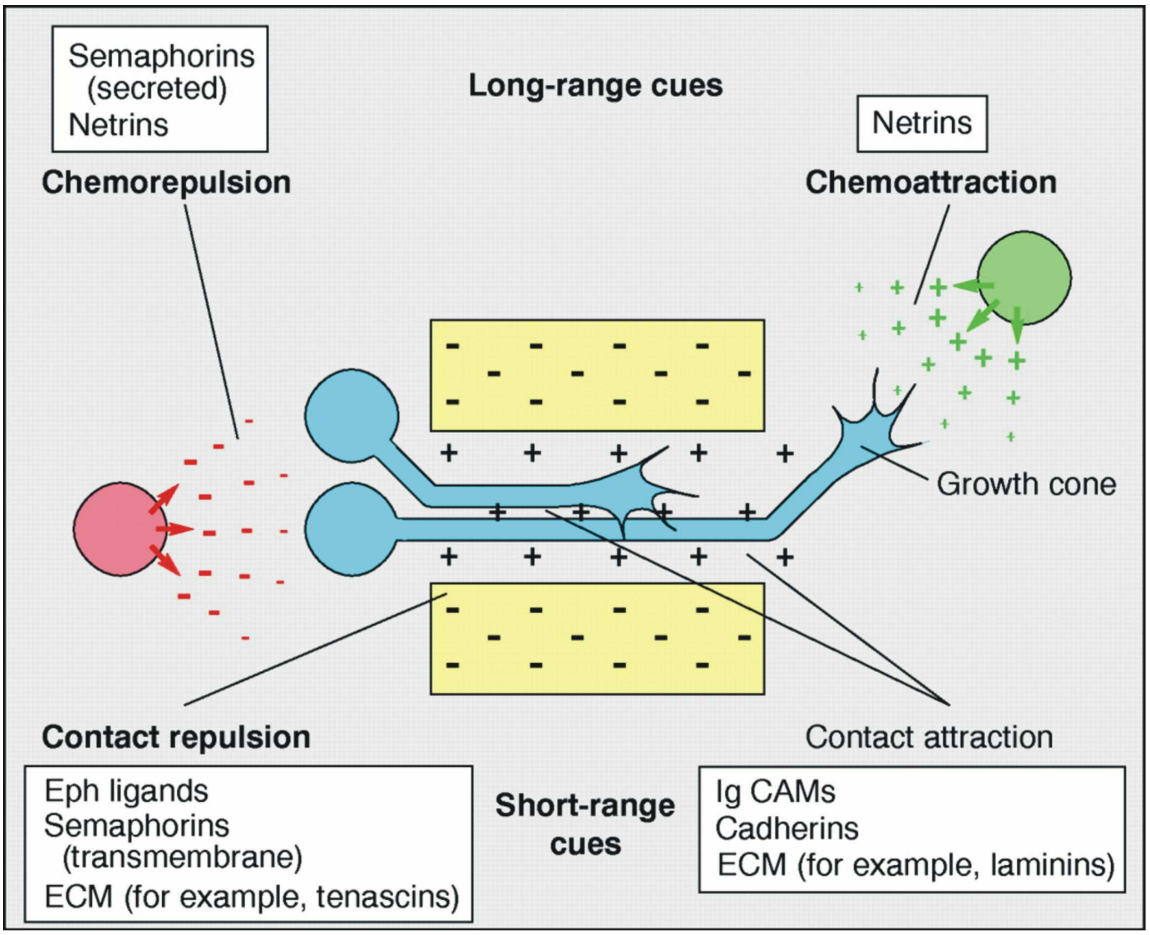


Figure 1. Guidance forces. Four types of mechanisms contribute to growth cone guidance: contact attraction, chemoattraction, contact repulsion, and chemorepulsion. The term attraction is used here to refer to a range of permissive and attractive effects, and the term repulsion to a range of inhibitory and repulsive effects. Examples of ligands implicated in mediating each of these mechanisms are provided in boxes. There is not a one-to-one match between molecules and mechanisms because some guidance molecules are not exclusively attractive or repulsive, but rather bifunctional, and some families of guidance cues have both diffusible and nondiffusible members. Individual growth cones might be "pushed" from behind by a chemorepellent (red), "pulled" from afar by a chemoattractant (green), and "hemmed in" by attractive (gray) and repulsive (yellow) local cues. Axons can also be guided by cues provided by other axons (selective fasciculation). Push, pull, and hem: these forces act together to ensure accurate guidance (From Tessier-Lavigne and Goodman, 1996).

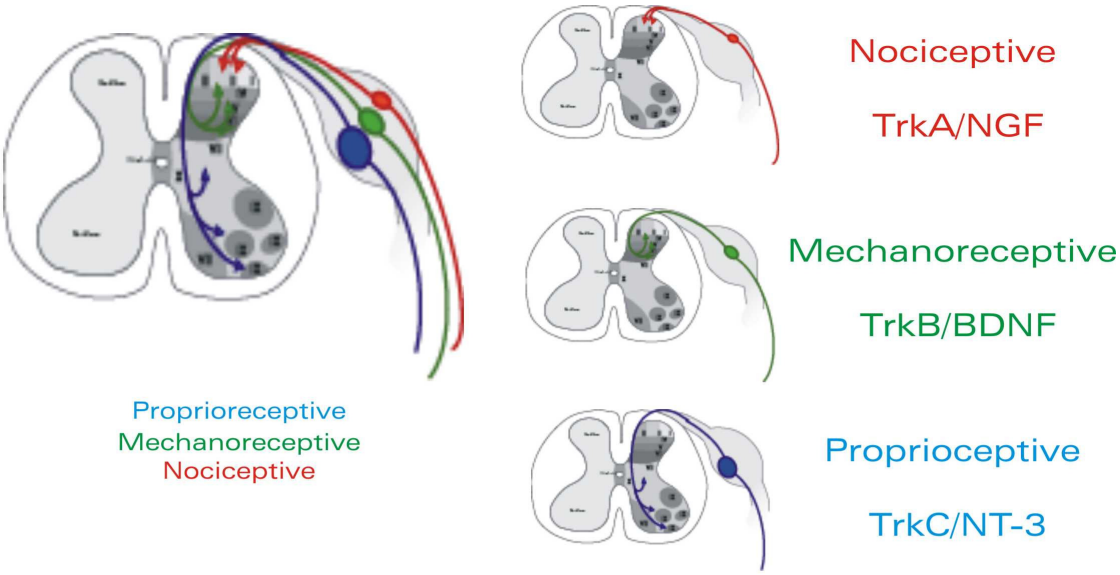


Figure 2. Dorsal root ganglia contain different subpopulations of sensory neurons that extent their axons to different laminae of the spinal cord and innervate specific peripheral targets. In rodents the nociceptive afferents terminate in the dorsal-most laminae I and II, mechanoreceptive afferents terminate more ventrally between laminae III and VI, while proprioceptive afferents reach the ventral horns where they form synapses with the motorneurons. The same organization is maintained in the avian spinal cord with the only difference that laminae I-III are laterally to medially oriented rather than dorsally to ventrally. The mechanism and guidance cues responsible for the modality-dependent targeting of the central afferents of the sensory neurons remain unknown

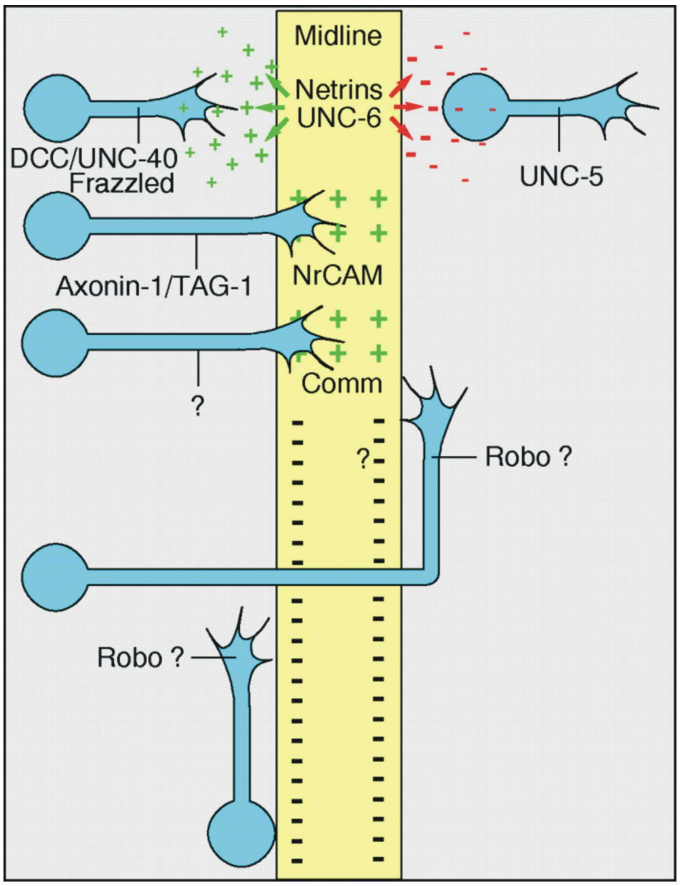


Figure 3. Long-range and short-range guidance at the ventral midline. A composite picture of guidance at the midline including mechanisms identified in nematodes, fruit flies, and vertebrates, at least some of which (and possibly all of which) are conserved among these organisms. The netrins appear to function as both long-range chemoattractants (green) and chemorepellents (red) for distinct classes of axons. Attraction of growth cones by netrins involves the DCC/UNC-40/Frazzled receptor (as shown in all three phyla), whereas repulsion of growth cones by netrins involves the UNC-5 receptor (as shown in C. elegans). In chick, crossing of the midline requires interaction of the Ig CAM axonin-1/TAG-1 on commissural axons with NrCAM on the surface of midline cells. In Drosophila, it also requires the midline expression of Commissureless (the growth cone receptor for Comm is at present unknown). After crossing many commissural growth cones turn longitudinally along the midline. In Drosophila, molecular data showed that the post-commissural axons express the Robo receptor that binds to the chemorepellent Slit which is secreted from floor-plate cells. The robo-slit interaction prevents commissural axons to recross the midline (From Tessier-Lavigne and Goodman, 1996)

2. Materials and Methods

2.1 Preparation of tissue sections

Transverse sections of chicken embryos of different developmental stages were used for protein detection, with immunohistochemistry, and RNA detection with *in situ* hybridization. In both cases the sections were collected in the same manner. Fertilized eggs were incubated at 37-39^{o}C for an appropriate period of time. Embryos were sacrificed by decapitation and pinned down in a Petri dish filled with Phosphate Buffered Saline (PBS). Subsequently, the embryos were staged according to the features described by Hamburger and Hamilton (1951). All internal organs were removed so that the spinal cord and the DRGs were visible. PBS was replaced with 4% Paraformaldehyde for tissue fixation. Depending on the stage of the embryo the duration of tissue fixation varied from 1 hour at room temperature to overnight at 4 ^{o}C. After fixation, tissue was cryoprotected with an overnight incubation in 25% sucrose/PBS solution at 4 ^{o}C, and embedded in OCT for freezing in isopentane cooled on dry ice. In most cases 20 μm-thick transverse sections of the lumbar region were collected on SuperFrost Plus microscope slides (Menzel-Glaeser), dried for 3 hours at room temperature and stored at -20^{o}C until further use.

2.2 Immunohistochemistry

a. On sections

Air-dried cryostat sections were transferred to a glass cuvette for a 3 min incubation in prewarmed PBS, followed by rinsing in cold H_2O to remove the OCT. Sections were incubated in 20mM lysine/0.1M sodium phosphate buffer, adjusted to pH 7.4, for 30min followed by two 10min washing in PBS. Tissue was permeabilized during a 30min incubation in 0.1% (v/v) Triton X-100/PBS at room temperature. In most of cases the blocking buffer was 10% fetal calf serum (FCS)/PBS supplemented with 0.2% (w/v) milk powder and 0.1% (v/v) Tween-20, with slight variations

depending on the antibody used. After 1 hour of blocking at room temperature, the primary antibody diluted in blocking solution was added and the sections were incubated overnight at 4 ^{o}C. Subsequently, the primary antibody solution was aspirated and sections were washed three times in PBS for 10 min, followed by incubation in blocking solution for 1 hour at room temperature. To detect the antigen, secondary antibodies with a binding specificity for the primary antibody, conjugated with a fluorescent probe, were added to the sections and incubated for 90 min at room temperature. To avoid bleaching the sections were kept in the dark from this step onwards. The secondary antibody solution was tipped off and the sections were washed for an hour with 5 changes of PBS. Finally, the sections were mounted with Immunomount, sealed with nail polish and analysed under a fluorescent microscope.

b. Whole mounts

For whole-mount staining embryos were sacrificed, staged, dissected and fixed as described above. Following rinsing in PBS, the embryos were transferred to 24well-plates and permeabilized with 1% (v/v) Triton X-100 in PBS for 1 hour at room temperature with constant agitation. After rinsing them in PBS, the embryos were incubated in 20mM lysine/0.1M sodium phosphate (pH 7.3-7.4) for 1 hour and washed three times in PBS for 30 min. Unspecific antibody binding was reduced with a 2-hour incubation in 10% (v/v) FCS/PBS. The embryos were then incubated for 48 hours at 4 ^{o}C with the primary antibody diluted in blocking solution. Following an extensive wash in PBS overnight, the embryos were incubated for 2 hours in blocking solution prior to the addition of the secondary antibody diluted in blocking solution. All the additional steps were performed in the dark to avoid bleaching of the fluorescent tag conjugated to the secondary antibody. Embryos were incubated 2 hours in

the secondary antibody solution, washed for 2 hours in PBS with a minimum of 5 changes.

To increase the visibility of the fluorescent staining we cleared the tissue. For this purpose, embryos were dehydrated by 10min sequential incubations in 25%, 50%, 75% and 100% methanol. The embryos were transferred to glass tubes and the tissue was cleared with the addition of BBBA (benzyl benzoate/ benzyl alcohol 2:1). The tubes were gently agitated until the embryos were translucent and ready for analysis with fluorescent microscopy.

2.3 *In situ* hybridization

To localize mRNA we have used *in situ* hybridization on transverse sections, prepared as described above.

a. Preparation of *in situ* probes

In situ probes were prepared with *in vitro* transcription using either plasmids where the appropriate sequence has been inserted into or purchased ESTs (for detailed information refer to the table within this section). Ten μg of purified plasmid were linearized with the appropriate restriction enzymes with restriction sites present in the vector at the 5' prime or 3' end of the cloned sequence. Complete linearization of the plasmid was verified with gel electrophoresis. Subsequently, the DNA was extracted with phenol:chlorophorm:isoamylalcohol (25:24:1). DNA present in the aqueous phase of the extraction was precipitated with the addition of 2.5 volumes of ethanol and 0.1 volumes of 3 M sodium acetate. After an overnight incubation at -20 ^{o}C the linearized plasmid DNA was spun down for 20 min at 14000 rpm, the pellet was washed with 70% ethanol in diethylpyrocarbonate (DEPC) treated H_2O and finally dissolved in DEPC- H_2O.

To generate the *in situ* probes, 1 µg of appropriately linearized plasmid was used as a template for *in vitro* transcription according to the following protocol:

2µl linearized plasmid (1µg)
0.8µl RNasin (purchased from NEB)
3µl DIG RNA Labelling Mix (purchased from Roche)
3µl DTT (1µg/ml) (purchased from Promega)
3µl 5x Transcription Buffer (purchased from NEB)
3µl T7 or SP6 polymerase
up to 30µl DEPC- H_2O

The *in vitro* transcription mix was incubated at 37^{o}C for 3 hours. An additional incubation with 1 µl DNase I (Invitrogen) at room temperature for 1hour removed the template DNA and the RNA probes were extracted with phenol:chlorophorm:isoamylalcohol. To the aqueous phase 2.5 volumes of ethanol and 0.1 volume of ammonium acetate (7.5M) were added. The samples were incubated overnight at -70^{o}C and spun down for 30 min at 14000 rpm. The pellet was washed with 70% ethanol/DEPC-H_2O, air-dried, dissolved in DEPC- H_2O and stored at -70 ^{o}C until further use.

b. Preparation of sections and hybridization

The first day of *in situ* hybridization should be performed in an RNase-free environment and all the solutions used should be treated with DEPC in order to avoid contamination with RNases that would degrade the RNA probes.
Sections were fixed in 4% (w/v) PFA/DEPC-PBS for 30 min at room temperature, washed twice with DEPC-PBS for 5 min and once with DEPC-H_2O. Acetylation was carried out in 1% (v/v) triethanolamine (Sigma) and 0.25% (v/v) acetic anhydride (Sigma) in DEPC- H_2O. The sections were washed twice with PBS-DEPC for 5min at room temperature, followed by a

washing step in 2xSSC (0.3 M sodium chloride, 30 mM tri-sodium citrate in DEPC-H_2O) for another 5 min at room temperature. For prehybridization 400 µl of prehybridization solution (50% formamide, 5xSSC, 5xDenhardts, 250µg/ml yeast total RNA, 500µg/ml herring sperm in DEPC- H_2O) were added per slide. Slides were covered with parafilm to avoid evaporation, placed in a hybridization box containing tissues soaked in 50% formamide/5xSSC and placed in a hybridization oven (Hybaid) at 56°C for 3 hours. The prehybridization buffer was tipped off the slides and hybridization buffer, containing the appropriate amount of RNA probe, was added on the sections. Subsequently, the sections were covered with parafilm, placed in the hybridization box and incubated overnight at 56°C. The concentration of the sense and antisense probe varied from 0.1-1 µg per slide depending on the specificity of each individual probe.

After hybridization, sections were washed twice in 5xSSC for 5 min. The slides were consecutively washed in 2xSSC for 5 min, 0.2xSSC for 5 min and in 50% Formamide/0.2xSSC for 20 min. All washing steps were performed at 56°C and the solutions used were prewarmed at 56°C. The following washing steps were carried out at room temperature. The sections were washed in 0.2xSSC for 5 min and twice for 5 min in detection buffer (0.1 M Tris-base, 0.15 M sodium phosphate dissolved in UHP-H_2O, pH adjusted to 7.5) and incubated in blocking solution (3% milk powder in detection buffer) for 60 min. After blocking the sections were placed in the hybridization chamber and incubated for 2 hours at room temperature with anti-DIG solution (1:5000 Anti-DIG antibody (Roche) in blocking solution). Subsequently, sections were washed three times with detection buffer for 10 min and equilibrated with AP buffer (0.1 M Tris-base, 0.1 M sodium chloride dissolved in UHP-H_2O, pH adjusted to pH 9.5). Finally, 500 µl developing solution [10 ml AP buffer containing 100 µl Levamisole solution (24mg/ml in AP buffer), 45 µl NBT solution (75mg/ml in 70% dimethylformamide) and 35 µl X-Phosphate solution (50mg/ml) were added per slide and incubated in the dark at room temperature until a signal was detectable. The development was stopped by

washing the slides twice for 10 min with Tris-EDTA. Sections were mounted with Cellvol mounting medium (polyvinylalcohol and sodium azide) and the expression pattern was analyzed with bright-field microscopy.

2.4 Preparation of double-stranded RNA

For the preparation of dsRNA molecules the same protocol described above for the preparation of *in situ* probes was used with the only difference that unlabelled rNTPs (25mM each) were used instead of the DIG labelling mix. The concentration of the sense and antisense ssRNA was measured with a spectrophotometer and equal amounts of each (200µg) were mixed and heated to 95^{o}C for 5 min. To allow for slow cooling, the heating block was switched off and the ssRNA probes were let to anneal for a minimum of 3 hours or until the temperature of the heating block dropped to 25 ^{o}C. The annealing efficiency was checked by gel electrophoresis as the dsRNA molecules have double the size of the non-annealed ssRNA molecules. Double-stranded RNAs were stored at -70 ^{o}C until further use.

2.5 *In ovo* injection and electroporation of dsRNA

Fertilized eggs were set at 37-39 ^{o}C and the embryos were let to develop for three days. To gain access to the embryo a “window” was opened in the egg shell and the embryo was staged according to Hamburger and Hamilton (1951). The extraembryonic membranes were removed with autoclaved dissection tools to avoid contamination of the embryo. A volume of approximately 1µl of dsRNA solution (concentration ranging 100-300ng/µl) was injected into the spinal cord of the developing embryo. The solution contained 0.1 volume of Trypan blue, that allowed visualizing the distribution of the solution during injection, and 200ng/µl of a reporter plasmid that drives YFP expression, were added to allow for the assessment of injection and electroporation efficiency. After adding a few drops of sterile PBS the embryos were electroporated in ovo with 5 square pulses at

25mV with a duration of 50msec (BTX Electroporator). After electroporation few drops of sterile PBS were added to cool down the embryo, the eggs were sealed with melted parafin and a coverslip or alternatively with scotch tape and they were returned to the incubator until the desired developmental stage was reached.

2.6 Analysis of commissural axon growth and guidance

The analysis of commissural axon trajectories was carried out as described previously (Perrin and Stoeckli, 2000; Stoeckli and Landmesser, 1995). Briefly, embryos were sacrificed between stages 25 and 26. The spinal cord was dissected out of the embryo, opened at the roof plate ("open-book preparation"), pinned down on a plate covered with Sylgard and fixed. To visualize the trajectories of the commissural axons the lipophilic dye Fast-DiI (5mg/ml in methanol; Molecular Probes) was injected to the dorsal part of the spinal cord to avoid labelling more ventrally located subpopulations of commissural neurons. The axonal trajectories of control, control-injected and experimental embryos were analyzed with confocal microscopy.

2.7 Grafting of hybridoma cells

The hybridoma cell line 5E1 developed by T.M. Jessell (Erickson et al., 1996), 1E8 developed by E. Frank (Bhattacharyya et al., 1991), and 9E10 developed by J.M. Bishop, were obtained from the Developmental Studies Hybridoma Bank from the University of Iowa. The cell line 5E1 produces a function-blocking anti-Shh antibody. The cell line 9E10 producing an antibody against c-myc, and 1E9 producing an antibody against P0 were used as controls. Cells were grown in DMEM/F12 supplemented with 10% FCS. Cells in exponential growth phase were pelleted by centrifugation and resuspended in PBS. Aliquots were taken for viability tests with Trypan Blue and cell counting. Hybridoma cells were injected into the lumbar level of stage 20 embryos *in ovo*. A peroxidase-coupled

secondary antibody (rabbit anti-mouse IgG, Cappel) was used to test for anti-Shh antibody production *in situ*. For this purpose open-books were dissected from stage 26 embryos, fixed in 4% PFA for 30-60 min and incubated with the secondary antibody diluted 1:500 in 10% FCS/PBS for 90 min at room temperature. Comparable numbers of cells from the 1E8 or the 9E10 hybridoma cell lines were grafted in control embryos.

2.8 Ectopic expression of *SHH*

For gain-of-function experiments the open reading frame of chicken *SHH* was amplified with RT-PCR and cloned into the pMES plasmid (kindly provided by C. Krull) using EcoRI restriction sites. Additionally, the *SHH* open reading frame was cloned into an expression vector derived from the pIRES plasmid (Clontech). The CMV promoter of the pIRES plasmid was replaced by the chicken *ACTB* promoter and the IRES sequence was removed. The *SHH* open reading frame was inserted in the expression vector using the *NheI* and the *SalI* restriction sites present in the multiple cloning site of the vector. The same restriction sites were introduced to *SHH* during the RT-PCR amplification of the open reading frame. To localize transfected cells and to assess the efficiency of injection and electroporation, we coinjected a plasmid encoding YFP. In the case of the pMES expression vector the pIRES sequence, downstream of the multiple cloning site, is followed by EGFP, allowing for direct detection of the transfected cells. To achieve a reversal of the endogenous Shh gradient, we used either shorter electrodes (2 mm rather than 4 mm) or we placed the commonly used electrodes more rostrally, such that floor-plate cells at more caudal levels of the lumbrosacral levels of the spinal cord were not transfected. The density of cells expressing either YFP or EGFP (depending on the expression vector used) was used to determine the electroporated area of the spinal cord and to distinguish between sites with high and low Shh expression.

2.9 *In vitro* assay

Open-book preparations of the lumbrosacral spinal cord of stage 24-25 embryos (Hamburger and Hamilton, 1951) were cultured in collagen gels essentially as described. However, we used the lipophilic dye Fast-DiI to label postcommissural axons rather than an anti-axonin antibody, as axonin-1 (the ortholog of rat TAG-1) is expressed also by motor neurons of the lumbrosacral spinal cord. As described previously for two-dimensional explant cultures of chicken commissural axons (Stoeckli et al., 1997), we used a serum-free medium to grow postcommissural axons in collagen gels. Heparin acrylic beads (Sigma) were soaked in either 0.5 mg/ml recombinant human Shh (RandD, with 25mg/ml bovine serum albumin as carrier), or 25 mg/ml bovine serum albumin (Albumax, Invitrogen) for 1 hr. Shh or control beads were positioned 200-700 µm from the explants. Cultures were grown for 24-36 hr before inspection on an inverted microscope. Explants with only very few axons entering the collagen gels or explants in direct contact with the bead were excluded from further analysis.

2.10 Purification of IgG from hybridoma serum

Monoclonal antibodies against Shh (5E1) were produced from the respective hybridoma cell lines purchased from the Developmental Studies Hybridoma Bank (DSHB). IgG purification from the supernatant of hybridoma cell cultures was performed with column chromatography. Protein A is a cell surface protein of bacterial origin that has a specific affinity to immunoglobulins.

The supernatant collected from the hybridoma cell cultures was purified by centrifugation and subsequent filtration through 0.22 µm pore size filters to remove all precipitates and cell debris to avoid clogging of the column. The high affinity protein extraction column was washed with water in order to remove the ethanol, in which the column is stored, and was equilibrated with Buffer A for at

least 10 column volumes. An additional washing with 0.1 M Glycine (Buffer B) removed all previously bound proteins from the column. Loading conditions were again restored with Buffer A. The washing steps were performed with a flow-rate of 20 ml/min.

Purified supernatant was loaded onto the well-equilibrated column with a flow-rate of 0.7 ml/min to allow IgG binding to the column. The unbound and unspecifically bound proteins were washed from the column with at least 10 volumes of Buffer A. The 5E1 IgGs, bound to the column, were eluted with a 5 min incubation of the column with Buffer B. Elution was continued and IgG samples were collected for the time period that the absorption of the spectrophometer was above the base line. The collected samples were neutralized immediately after elution and the column was equilibrated with a washing with Buffer A. With this approach 2 ml of 0.8 mg/ml of 5E1 antibody were collected. The specificity of the purified 5E1 antibody was checked with immunohistochemistry and western blotting.

2.11 Embryonic surgery and neurotrophin treatments

Fertile eggs were windowed on the third day of incubation [embryonic day (E)3, stage 17-18; Hamburger and Hamilton, 1951], and the right limb bud was removed at stage 20-21, using flame-sharpened tungsten needles. The embryos were moistened with several drops of filter-sterile PBS, closed with a coverslip and returned to the incubator for 36-48 hr. Beginning at stage 24-25, when sensory neurons would normally begin to invade the limb (Tosney and Landmesser, 1985), surviving embryos were treated twice daily with 10 µg/ml human recombinant BDNF (Alomone) or human recombinant NT-3 (Alomone) or natural murine NGF (Life Sciences) by injection at the base of the limb bud. After 3 treatments, the surviving embryos were killed on E7 at stage 32-33. The embryos were washed with PBS, decapitated and eviscerated. Only those embryos observed to have a complete absence of the leg and pelvic girdle were used for further analysis. These embryos were then processed for either

immunohistochemisry (see respective paragraph) or dorsal root ganglion removal (see respective paragraph).

2.12 Cell counts

Control, limb-ablated, and limb-ablated embryos that received injections of neurotrophins were removed from the egg shell, decapitated and immediately placed in 4% PFA solution. After overnight fixation at 4^{o}C the embryos were incubated for two days in 25% sucrose solution, embedded in OCT, sectioned serially along the lumbar region at 20 μm, and processed for TUNEL staining (see respective paragraph), immunohistochemistry with anti-NeuN antibody (dilution 1:500; Chemicon) or DAPI staining. DRG sensory neurons and pyknotic cells were counted in every third section through the entire lumbar enlargement.

2.13 TUNEL staining

For the detection of apoptotic cells we have used the *in situ* cell death detection kit (Roche Diagnostics) according to manufacturer instructions. Briefly, transverse sections of cryopreserved stage-32 chicken embryos that have been limb ablated were incubated for 3 min in prewarmed (37^{o}C) PBS so as to remove the OCT. Subsequently, the sections were fixed with 2% PFA at room temperature for 20 min, washed three times with PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 1 hr.

To detect the apoptotic cells the sections were washed twice with PBS for 10 min at room temperature and 50 μl of reaction mixture was added per section, the sections were covered with parafilm and incubated at 37^{o}C in a humidified chamber for 1 hr. Finally, the sections were rinsed twice with PBS and mounted with Immunomount (Shandon). Apoptotic cells were observed with fluorescence microscopy.

2.14 Dorsal root ganglion removal

Limb-ablated and neurotrophin-treated embryos (see previous paragraph) were sacrificed at stage 32-33. The lumbosacral regions of the spinal cord were exposed by a ventral laminectomy that facilitated the removal of the DRGs facing the limb ablated side of the embryo. Using tungsten needles 3 to 4 DRGs of the lumbosacral level were removed and were frozen in liquid nitrogen. Approximately 250 lumbar DRGs from 70 embryos per treatment were collected.

2.15 Total RNA extraction and subtractive library production

The frozen tissue was processed for total RNA extraction with the RNeasy Mini Kit (Qiagen) according to manufacture guidelines. Since the amounts of total RNA obtained with this approach were limiting we have used the Super SMART PCR cDNA synthesis kit (BD Biosciences) that allows cDNA amplification. The resulting cDNAs were subsequently used for the subtractive screens with the PCR-Select cDNA subtraction kit (Clontech) according to manufacturer´s guidelines (Duguid and Dinauer, 1990). Differentially expressed cDNAs were subsequently T/A cloned in the pDrive Cloning Vector included in PCR cloning kit (Qiagen) according to manufacturer's instructions.

3. Results

3.1 Characterization of Shh as a guidance cue for the commissural axons

3.1.1 Isolation of candidate guidance cues directing commissural axons along the longitudinal axis of the spinal cord

In order to identify candidate guidance cues that direct commissural axons rostrally after their midline crossing, we used a subtractive hybridization approach to search for differentially expressed floor-plate genes. The assumption that such guidance cues were expressed in the floor plate was based on the finding that commissural axons turn in close contact with the floor-plate border (Bovolenta and Dodd, 1990) and also on the failure of the axons to turn into the longitudinal axis in the absence of a floor plate in mouse (Bovolenta and Dodd, 1991; Matise et al., 1999), in chick (van Straaten and Hekking, 1991), in zebrafish (Greenspoon et al., 1995; Hatta, 1992) and in Xenopus (Clarke et al., 1991). All of the analyses described further were performed only at the lumbrosacral level of the spinal cord so as to exclude variations due to timing or changes in the pathfinding behaviour between different subpopulations of commissural neurons. At stage 25, the majority of the commissural axons at the lumbrosacral level have turned into the longitudinal axis, thus we expected the putative guidance cues to be expressed at its highest levels (**Fig. 4**). On the other hand at stage 20, commissural axons have just started to grow in the dorsal spinal cord and have not yet reached the floor plate. Therefore, we expected that at this stage the guidance cues mediating the rostral turning of the commissural axons along the longitudinal axis might not be expressed at all, or at least only at low levels. As the amount of starting material was very limited we used a PCR-based approach for subtractive hybridization (for further details see experimental procedures). Initially, we obtained several hundred clones from which 400 were randomly selected for further analysis. After eliminating false positive clones by forward and reverse hybridization we selected 30 clones with a

clear difference in expression level between stages 20 and 26. The selected differentially expressed cDNAs had a size range from several hundred to more than a thousand base pairs. The cDNAs were used directly for the synthesis of DIG-labelled *in situ* probes as well as dsRNA used for functional analysis.

3.1.2 Candidate clones were selected based on their expression pattern

As a first step, we assessed the expression pattern of the candidate genes in the spinal cord by *in situ* hybridization. For further analysis, we concentrated on those clones that were predominantly restricted to the floor plate during the relevant time window i.e stage 20 to 26. The selected cDNA fragments were sequenced and compared to sequences at the NCBI data bank. One of the clones was identified as F-spondin (Klar et al., 1992; Burstyn-Cohen et al., 1999). For several of the obtained clones no entries were found in the data bank presumably because we had sequence information from the 3'-untranslated region. Full-length sequences of our candidate clones were not required for further analysis, as the isolated cDNA fragments from identified candidate genes could be used directly for functional analysis with RNAi (Pekarik et al., 2003).

3.1.3 Funtional analysis of candidate guidance cues with *in ovo* RNAi

The injection of dsRNA derived from the cDNA fragments of candidate genes into the central canal of the spinal cord, in combination with *in ovo* electroporation, resulted in specific downregulation of the targeted genes (Bourikas and Stoeckli, 2003; Pekarik et al., 2003; Stoeckli, 2003). Using this assay, we found that one of our candidate genes indeed interfered with the decision of postcommissural axons to turn rostrally (**Fig. 5A,B**). We did not detect any effects on axon growth and pathfinding toward and into the floor plate area; however, most axons lingered at the exit site from the floor plate. The majority of the growth cones did not have the bias to grow in rostral direction or even pointed caudally. Relatively few axons extended along the longitudinal axis. Among those, many erroneously

turned caudally instead of rostrally. A total of 29 embryos were treated with dsRNA from the identified clone. On average, nine injections sites were analyzed per embryo. An abnormal phenotype (stalling with lack of rostral bias, caudal turn of axons, or both) was seen in 90% of the embryos and at least 78% of the injection sites per embryo. In age-matched control embryos (*n*=27), commissural axons did not linger at the floor-plate exit point (average of 9 injection sites per spinal cord). Even when commissural axons were analyzed in younger control embryos, axons never showed the lingering morphology seen in experimental embryos that is characterized by enlarged growth cones and/or orientation in any direction other than rostral. Therefore, we concluded that we had identified a guidance cue providing an instuctive signal for the growth along the longitudinal axis. In its absence, commissural axons either stalled at the floor-plate exit site or randomly chose in which direction to turn.

3.1.4 Shh identified as guidance cue for postcommissural axons

To identify the candidate gene that was shown to interfere with the rostral turn of postcommisural axons, we used the cDNA fragment from our screen as a probe to search a cDNA library derived from E14 chicken brain. The resulting cDNA was sequenced and found to encode Shh.

The role of Shh as a guidance cue for postcommissural axons was confirmed by *in ovo* RNAi with a second, non-overlapping fragment of dsRNA that was derived from the N-terminal part of the *SHH* cDNA (**Fig. 5C,D**). The efficiency of the approach depended on targeting the Shh dsRNA into the developing floor plate. We have injected a GFP-expressing vector and electroporated by positioning the electrodes dorsally and ventrally of the spinal cord with the positive electrode facing the ventral part (**Fig. 6A**). Downregulation of *SHH* was shown with immunohistochemistry on transverse sections of embryos sacrificed 2 days after the injection of *SHH* dsRNA (**Fig. 6C,D**) compared to age-matched controls (**Fig. 6G,H**).

The phenotypes obtained by *in ovo* RNAi using the two different dsRNA fragments were indistinguishable with respect to both phenotype reproducibility and penetrance. As shown before with the dsRNA derived from the 3'-UTR of *SHH,* we injected embryos at stage 18-19 (*n*=10) and analyzed them at stages 25-26. Eight of the ten embryos showed the abnormal phenotype at 92.5% of the injection sites (range 78-100%; average, 10 injection sites per spinal cord).

3.1.5 Blocking Shh confirms its role in axon guidance

As a different means to induce *SHH* loss-of-function phenotypes, we used function-blocking antibodies. In contrast to previous *in vivo* assays at the protein level (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Perrin et al., 2001), we did not only inject purified antibodies but we also engrafted hybridoma cells (clone 5E1) directly into the spinal cord. The interference with *SHH* function at the protein level resulted in the same phenotype that was observed after silencing *SHH* by *in ovo* RNAi (**Fig. 7A**). Commissural axons were no longer instructed to turn rostrally after midline crossing and therefore stalled at the floor-plate exit point or chose randomly to grow either rostrally or caudally. The phenotype was observed in eight of ten embryos injected with 5E1 antibodies (average 8 injection sites, 90% showing the indicated phenotype). Sixteen embryos received a graft of 5E1 hybridoma cells. As a control, hybridoma cells producing an antibody against c-Myc (9E10 cells) or against P0, an epitope not expressed in the spinal cord (1E8 cells), were used (*n*=5 and 4 embryos, respectively). None of the control embryos showed either stalling or caudal turns at any of the injection sites (9 or 10 injection sites per spinal cord; **Fig. 7B**).

3.1.6 Shh has a direct effect on postcommissural axons

Shh is well known for its effect on spinal cord patterning (Briscoe and Ericson, 1999; Briscoe et al., 2000; Wijgerde et al., 2002). It acts in a graded manner to establish different populations of neural progenitors, as defined by the expression

of homeodomain transcription factors that are either repressed (class I) or induced (class II) by Shh.

To exclude the possibility that the patterning of the spinal cord was changed by our interference with *SHH* function, thus leading to an indirect effect on postcommissural axon pathfinding, we analyzed the expression pattern of some class I and class II genes and compared them to control embryos. We did not detect any difference in spinal cord patterning with respect to *PAX7* (classI; **Fig. 8A,D**; Briscoe et al., 2000) or *ISL1* (**Fig. 8B,E**) and *NKX2.2* (Briscoe et al., 1999; **Fig. 8C,F**). Therefore, we concluded that *SHH* did not affect commissural axon guidance indirectly by changing cell differentiation or the patterning of the spinal cord. However, downregulation of *SHH* in an earlier time window (before stage 14) using the same dsRNA fragment did result in a decrease or even in the absence of *ISL1* and *NKX2.2* expression (at lumbrosacral levels; **Fig. 9A-D**). When *SHH* was downregulated by *in ovo* RNAi at stage 8, even HNF3β, a marker of floor-plate cells known to be a target of Shh signalling, was reduced, in line with the notion that Shh has a role in floor-plate induction as well as patterning of the spinal cord along the dorsoventral axis (Briscoe et al., 2000).

3.1.7 Shh's effect on axon guidance

To obtain further evidence for a direct effect of Shh on commissural axon guidance, we tried to identify its receptor on commissural neurons. In both invertebrates and vertebrates Patched (Ptc) and Smoothened (Smo) have been identified as co-receptors for Shh, mediating its inducing activities (Goodrich et al., 1996; Stone et al., 1996). Ptc is the binding module of the receptor of Shh while Smo acts as the signalling module. Upon binding of Shh, Ptc releases and derepresses Smo (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), which in turn triggers the signalling cascade (Ingham and McMahon, 2001).

To test whether Ptc and Smo were mediating Shh's effect on axon guidance, we looked at their expression in the developing chicken spinal cord (**Fig. 10B-F**). Throughout the relevant time window during which commissural axons cross the

floor plate and turn rostrally along the contralateral floor-plate border, neither *PTC* nor *SMO* are expressed by commissural neurons, suggesting that they are not required for *SHH* function in postcommissural axon guidance.

Because expression levels of Ptc are influenced by Shh (Goodrich et al., 1996; Ingham and McMahon, 2001), we also compared *PTC* expression by *in situ* hybridization between control embryos and embryos treated with *SHH* dsRNA (**Fig. 11A-D**). No changes in *PTC* and *SMO* expression in commissural neurons were detected at stage 25.

Additional evidence for a receptor other then the complex of Ptc and Smo was obtained in functional experiments using cyclopamine, a small molecular inhibitor of Smo (Incardona et al., 1998). The injection of cyclopamine between stages 19 and 20 did not cause any changes in the turning behaviour of commissural axons after midline crossing (*n*=16; data not shown). Furthermore, downregulation of *SMO* by *in ovo* RNAi did not interfere with commissural axon guidance (*n*=16 embryos; **Fig. 11E**) Therefore, we concluded that the effect of Shh on postcommissural axon guidance was not mediated by Ptc and Smo.

3.1.8 Shh's effect on postcommissural axons is mediated by Hip

Vertebrates, unlike invertebrates, express an additional receptor for Shh, called Hedgehog Interacting Protein (Hip). Hip is a transmembrane protein that has been shown to bind directly to all vertebrate hedgehog proteins: Indian, Desert and Sonic Hedgehogs (Chuang and McMahon, 1999). Because the distribution and functional evidence excluded Ptc and Smo as the Shh receptor involved in axon guidance, we analyzed the expression of *HIP* during the time window of commissural axon navigation (**Fig. 12A-C**). *HIP* was regulated very dynamically and expressed by very few cells any given time. Commissural neurons expressed *HIP* very transiently at stage 24, which is the time point at which axons have reached the contralateral floor-plate border and turn into the longitudinal axis. The expression of *HIP* in the spinal cord was not changed in response to silencing of *SHH* (**Fig. 13A,B**).

Direct evidence for an involvement of Hip as mediator of the Shh signal was found by *in ovo* RNAi. The electroporation was performed by placing the electrodes parallel to the spinal cord with the positive electrode on the right side of the embryo (**Fig. 14A**). The efficiency of this targeting approach was shown with the use of a reporter vector expressing GFP (**Fig. 14B-D**). *In situ* hybridization was applied, in the absence of a Hip specific antibody, to show downregulation after *HIP* dsRNA injection and electroporation. It is obvious that the *HIP* mRNA was significantly downregulated on the treated side of the spinal cord compared to the contralateral side (**Fig. 14E**). Downregulation of *HIP* did not alter the expression level of *SHH*, as shown with 5E1 immunohistochemistry in *HIP* dsRNA-treated embryos (**Fig. 14F**)

Perturbation of *HIP* function in commissural axons resulted in the same turning phenotype as seen after downregulation of *SHH* (*n*=19 embryos, phenotype at 95% of the injection sites; **Fig. 15A-D**). Thus, we concluded that Hip was the receptor that mediated the effect of Shh on postcommissural axons.

3.1.9 Graded *SHH* expression suggests a repulsive mechanism

The expression pattern of Shh in transverse sections of chicken embryos was compatible with a role in the rostral turning of commissural axons as it was expressed within the floor plate at the time window that the axons turned rostrally (**Fig. 16A-F**). However, a graded distribution of a guidance cue would be expected to explain a rostral turn of postcommissural axons at the lumbosacral level of the embryonic chicken spinal cord. Depending on the mechanism, higher expression of an attractive cue would be expected rostrally, whereas a repellent cue should be expressed at higher levels caudally. As shown in open-book preparations, the expression of *SHH* was higher caudally (**Fig. 16G,H**), suggesting that Shh was providing a repellent signal for commissural axons that was mediated by Hip.

3.1.10 *SHH* gain-of-function phenotype is consistent with a repulsive role

To provide evidence for a repulsive activity of Shh on postcommissural axons, we used *in ovo* electroporation to selectively express *SHH* in the thoracic and upper lumbosacral levels on one side of the spinal cord (*n*=23 embryos; **Fig. 17A-D**). In areas with high Shh expression, most commissural axons did not leave the floor plate and stalled at the exit site (**Fig. 17E,F**). Some axons encountering a reversed gradient upon floor-plate exit - that is, axons turning slightly caudally of the electroporated area – showed the expected pathfinding errors: they stalled or turned caudally to avoid high concentrations of Shh (*n*=20 embryos; **Fig. 17G-H**). As a control, commissural axons ipsilateral to the ectopic *SHH* expression were traced. As expected, no effect on their turning behavior was detected, as they grew through the area of high Shh levels before they expressed Hip, but encountered a normal Shh gradient upon floor-plate exit, that is, when they expressed Hip (**Fig. 17J**).

3.1.11 Shh's repulsive activity confirmed by *in vitro* experiments

Further evidence for a repulsive role of Shh on postcommissural axons was found in an explant assay similar to the one described previously for rat tissue (Zou et al., 2000). We cultured spinal cord explants from stage 24-25 embryos in collagen gels in the presence of beads soaked in Shh or control beads soaked in bovine serum albumin (**Fig.18**). To identify postcommissural axons, we labelled dorsal commissural neurons with the DiI in open-book preparations of dissected spinal cords before cutting and culturing explants (**Fig. 18A**). Unlabelled axons, therefore, represent predominantly motor axons (which grow well in collagen gels), unlabeled postcommissural axons from more ventral areas of the spinal cord, or dorsal postcommissural axons that were not labelled with DiI. No DiI – labelled axons were found contacting Shh-soaked beads (**Fig. 18B,C**). Control beads were neither attractive nor repulsive for postcommissural axons (**Fig. 18E,F**). Most often (89.5% of the explants; *n*=19), axons did not grow out of the

explant on the side facing a Shh-soaked bead, or turned away from the bead as soon as they entered the collagen gel (**Fig. 18B,C**). In one case the Shh bead was placed 0.9mm away from the edge of the explant (**Fig. 18D**). In this situation axons started to grow toward the bead but stalled or turned approximately 300 µm away from the bead.

Taken together, our results demonstrated that postcommissural axons avoided territories with high Shh both *in vivo* and *in vitro*, indicating that Shh acted as a repellent for postcommisural axons. The repulsive activity of Shh was mediated by Hip, in contrast to its attractive effect that was mediated by Ptc and Smo (Charron et al., 2003).

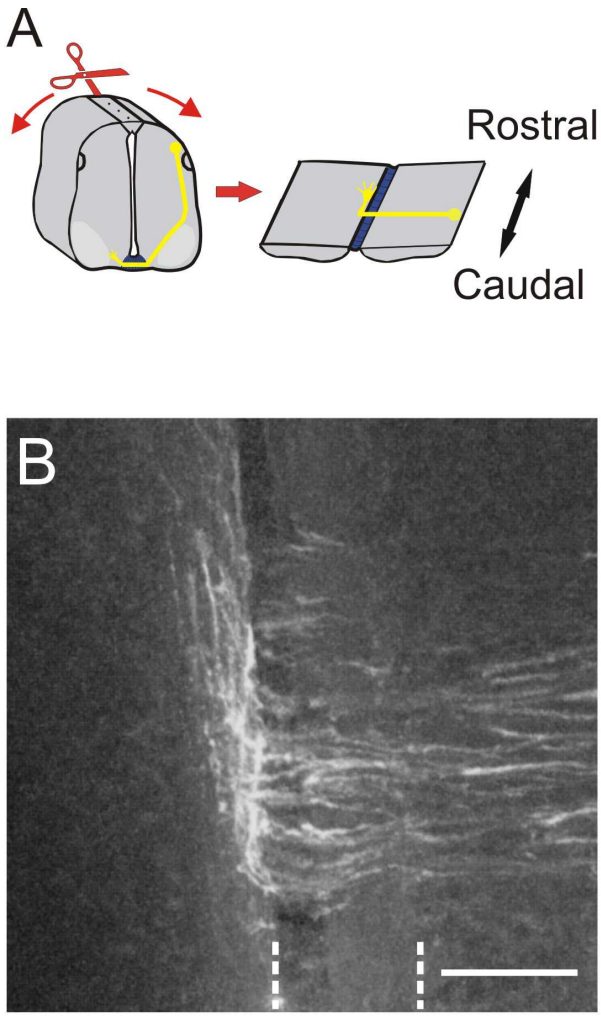


Figure 4 Commissural axons turn into the longitudinal axis of the spinal cord in close contact with the contralateral floor-plate border.

(A) Spinal cords were dissected and cut along the roof plate.

(B) The trajectory of commissural axons in a control embryo was visualized by application of FastDiI into the area of the cell body. The floor plate is indicated by dashed lines in B. Bar 100um
(Adapted from Bourikas et al., 2005)

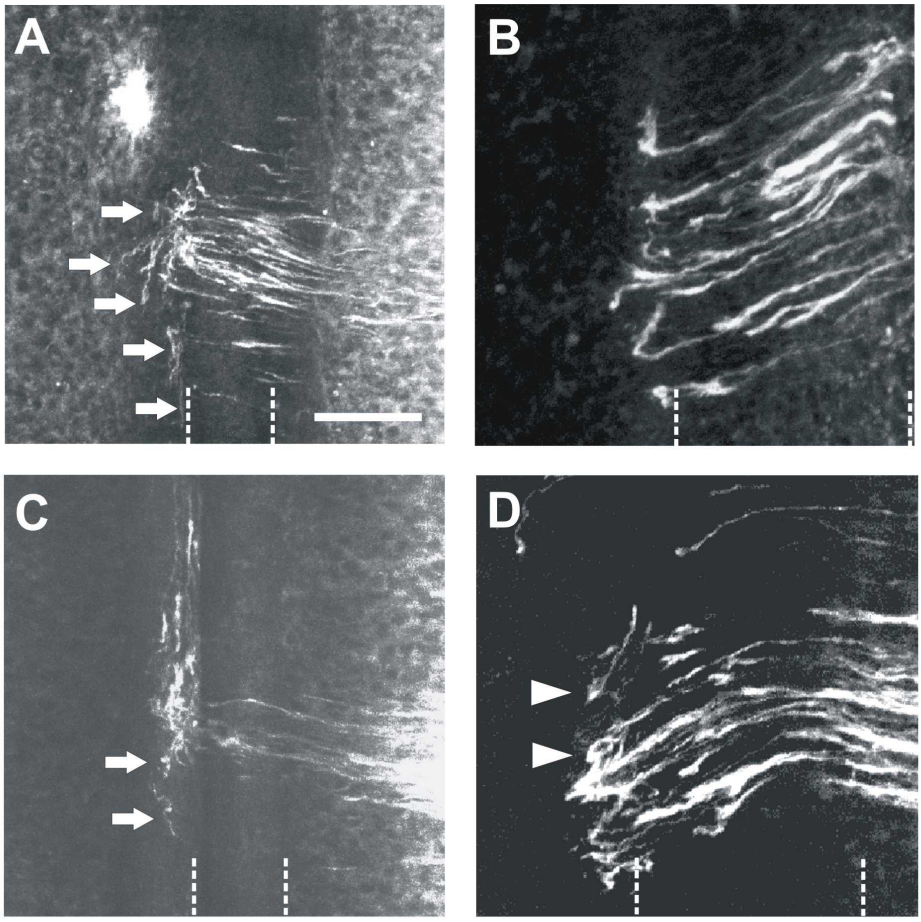


Figure 5 Shh directs postcommissural axons rostrally (**A-D**). Images of four chick embryos in which *SHH* function was abolished by in *ovo* RNAi. The phenotype reflecting the lack of *SHH* function was found with two independent fragments of *SHH* that were used for dsRNA production. One fragment was derived from the 3'-UTR of *SHH* (**A** and **B**) and the other from the fragment encoding the N-terminal part of Shh (**C** and **D**). Axons stalled at the contralateral floor-plate border or even turned caudally (arrows in **A** and **C**). Axons showed no difference in their growth toward, into, and across the floor plate as compared to controls (compare to **Fig. 4B),** but they did not turn rostrally along the contralateral floor-plate border. The majority of the axons stalled at floor-plate exit site (**A, B, D**). Growth cones clearly lacked the rostral bias that was seen in control embryos analyzed at earlier stages and explored movement in all possible directions (**B**). In some cases axons initially turned caudally but corrected their pathway by forming loops (arrowheads in **D**). Corrections of initial pathway choices were never seen in control embryos, in which axons were already biased in rostral direction upon floor-plate exit. The floor plate is indicated by dashed lines. Rostral direction is toward the top in all panels. Bar 100um in **A** and **C**, 30um in **B** and **D**. (Adapted from Bourikas et al., 2005)

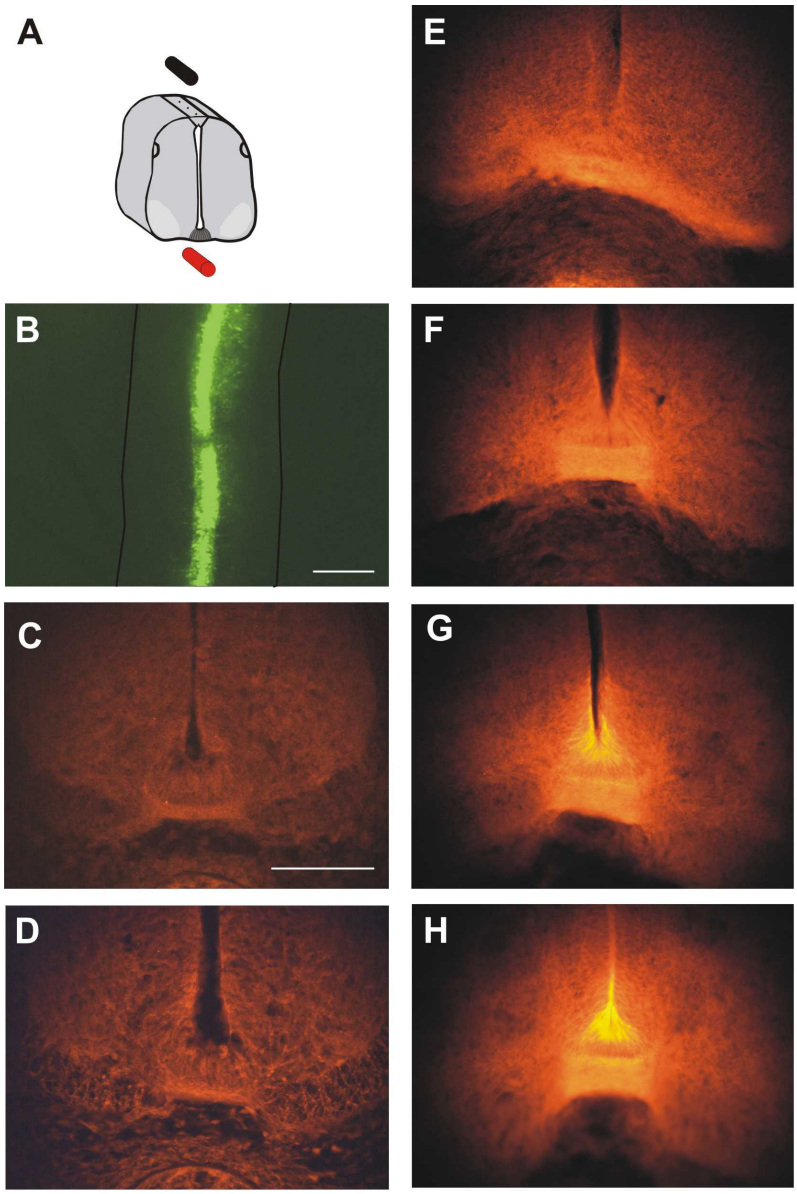


Figure 6 Downregulation of *SHH* by *in ovo* RNAi abolishes its increasing rostral to caudal gradient. In order to silence *SHH* by *in ovo* RNAi we injected dsRNA derived from *SHH* into the central canal of the spinal cord together with a plasmid encoding YFP to visualize transfected cells. Placing the electrodes over the dorsal (cathode) and the ventral (anode) midline of the spinal cord resulted in efficient targeting of floor-plate cells (**A,D**). Using this approach to silence *SHH* resulted in the almost complete absence of Shh protein (**C,D**). In a control embryo, Shh forms a gradient throughout the lumbosacral level of the spinal cord with lower levels rostrally (compare E to H). The sections shown in **C** and **D** correspond to **F** and **H**. Sections shown in **E-H** were taken every 100um. Sections from the control embryo and the embryo treated with *SHH* dsRNA were processed in parallel and imaged with the same camera settings. Bar 500um in **B**, 200um in **C-H**. (Adapted from Bourikas et al., 2005)

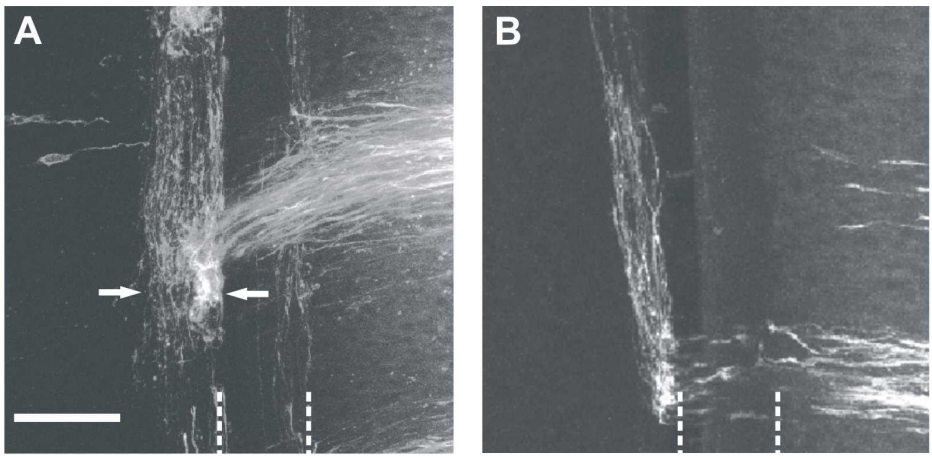


Figure 7 Blocking Shh function at the protein level confirms its role as a guidance cue for postcommissural axons. As an alternative method to demonstrate the role of Shh as a guidance cue for postcommissural axons, we injected 5E1 hybridoma cells into the central canal of the spinal cord. In accordance with the results from our *in ovo* RNAi experiments **(Fig.5),** the perturbation of Shh function at the protein level was found to interfere with the rostral turn of postcommissural axons **(A).** Axons either stalled at the floor-plate exit site or erroneously turned caudally (arrows in **A**). As a control, we used 9E10 hybridoma cells producing antibodies against c-myc. In this case there was no interference with commissural axon guidance. Dashed lines indicate the floor plate. Rostral is toward the top in both panels. Bar 100um. (Adapted from Bourikas et al., 2005)

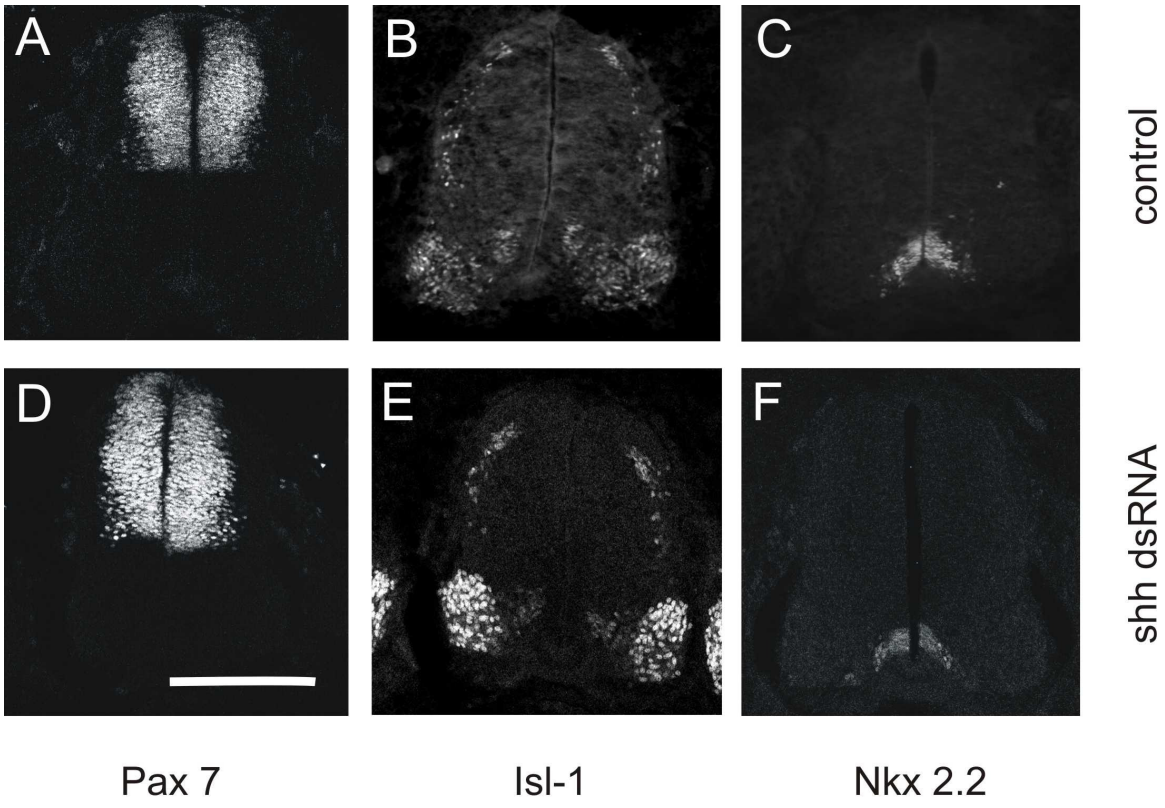


Figure 8 Perturbation of Shh function during the time window of commissural axon guidance does not interfere with spinal cord patterning. The morphogen Shh was shown to determine cell identity along the dorso-ventral axis of the spinal cord during early development (Briscoe and Ericson, 1999; Jessell, 2000; Lee and Pfaff, 2001). However, the interference with Shh function after the pattern of the spinal cord was established did not alter cell identity as determined by the expression of class I and class II transcription factors that are known to be either induced or repressed by Shh (see text for details). We chose *PAX7* as an example for a class I gene. Its expression pattern in dorsal spinal cord is similar at stage 23 (not shown) and stage 25 (**A-D**). As examples for class II genes, we used *ISL1* (islet-1) and *NKX2.2*. *ISL1* is expressed in motorneurons and a few cells of the dorsal spinal cord (**B-E**). *NKX2.2* is expressed in a small group of cells immediately adjacent to the floor plate (**C-F**). No difference in the expression of *PAX7 , ISL1* and *NKX2.2* was found between control embryos (**A-C**) and experimental embryos lacking Shh function (**D-F**), when *SHH* was silenced by *in ovo* RNAi at stage 18 or later. Bar 300um

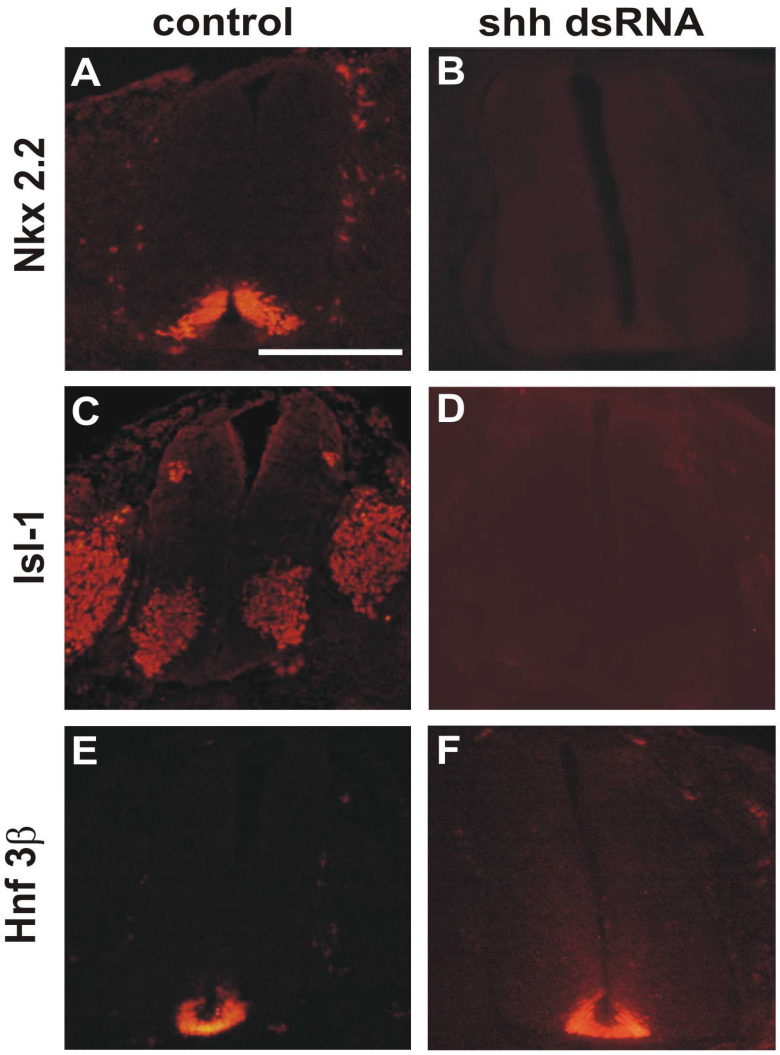


Figure 9 Silencing *SHH* during early spinal cord development interferes with patterning. As a positive control for *SHH* silencing by *in ovo* RNAi, we injected *SHH* dsRNA and electroporated chicken embryos between stage 13 and stage 14 (**B,D,F**) or at stage 8 (not shown). When compared to control embryos (**A,C,E**) the expression of selected markers of spinal cord patterning (see text for details), such as *NKX2.2* (**B**) or *ISL1* (**D**) was not induced indicating the absence of the normal dorso-ventral pattern of cell differentiation in the absence of Shh. *HNF3β* was still expressed in the floor plate of embryos treated with *SHH* dsRNA at stage 8 (not shown) albeit at reduced levels when compared to control embryos (**E**). No change in *HNF3β* expression was observed in embryos where *SHH* was silenced at stage 13 or older (**F**). These findings are in line with previous reports about the requirements for Shh during different time windows (Briscoe et al., 2000; Wijgerde et al., 2002; adapted from Bourikas et al., 2005)

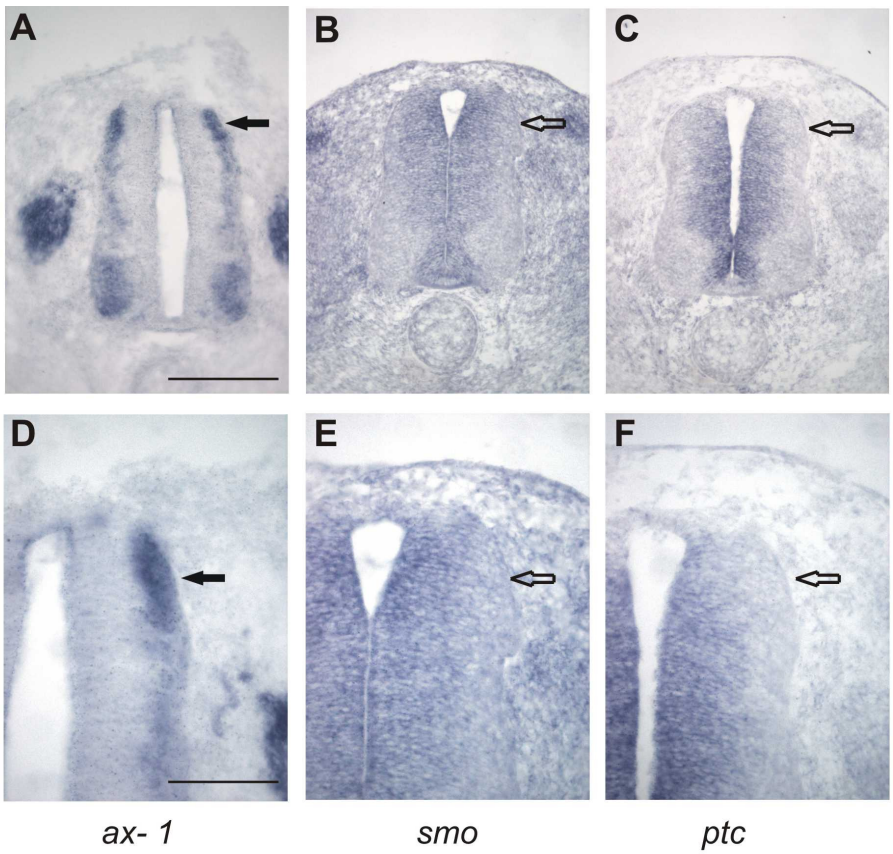


Figure 10 Neither Ptc nor Smo are expressed by commissural neurons when axons turn into the longitudinal axis (**A-C**). Transverse sections of stage 23 spinal cords shown at high magnification (**D-F**). Sections were used for *in situ* hybridization to demonstrate expression of Axonin-1 (**A** and **D**), Smo (**B** and **E**) and Ptc (**C** and **F**). Commissural axons at the lumbosacral level of the spinal cord cross the floor-plate at stage 23. By stage 24 they have reached the contralateral floor-plate border and turn rostrally into the longitudinal axis. During that time commissural neurons in the dorsolateral spinal cord expressed Axonin-1 (arrow in **A** and **D**) but they did not express Smo (open arrow in **B** and **E**) or Ptc (open arrow in **C** and **F**). The expression domains of both Smo and Ptc retracted to the ventricular zone. Bar, 200μm in **A-C**, 100μum in **D-F**. (Adapted from Bourikas et al., 2005)

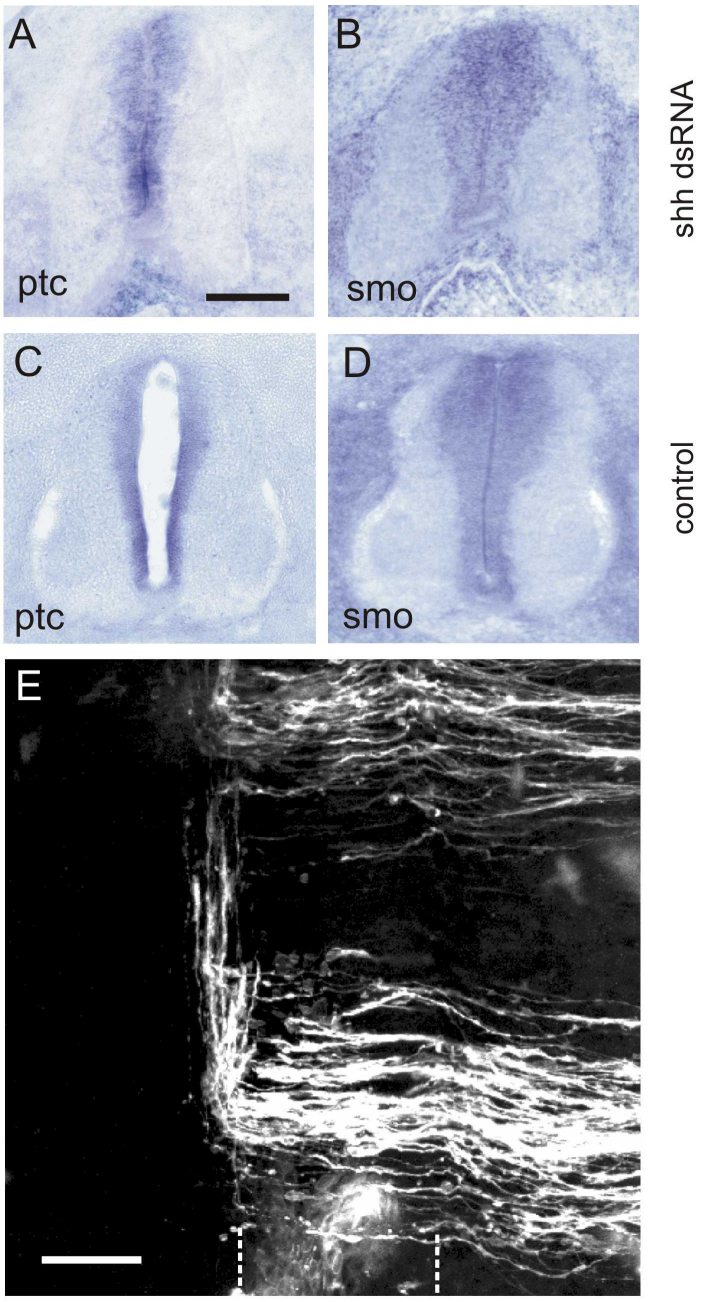


Figure 11 The effect of Shh on postcommissural axon guidance was not mediated by Ptc and Smo. Because Shh was shown to affect the expression level of Ptc (Goodrich et al., 1996), we compared the expression of Ptc and Smo in the spinal cord of embryos at stage 25 after silencing Shh by *in ovo* RNAi (**A,B**) with control embryos (**C,D**). Neither the pattern of Ptc (**A,C**) nor that of Smo (**B,D**) was changed after silencing *SHH*. Furthermore, silencing of *SMO* by *in ovo* RNAi (**E**) or by the injection of the alcaloid cyclopamine (not shown) did not change the pathfinding behavior of postcommissural axons. Dashed lines indicate the floor plate. Rostral is top in **E**. Bar 200μm in **A-D**; 50μm in **E**. (Adapted from Bourikas et al., 2005)

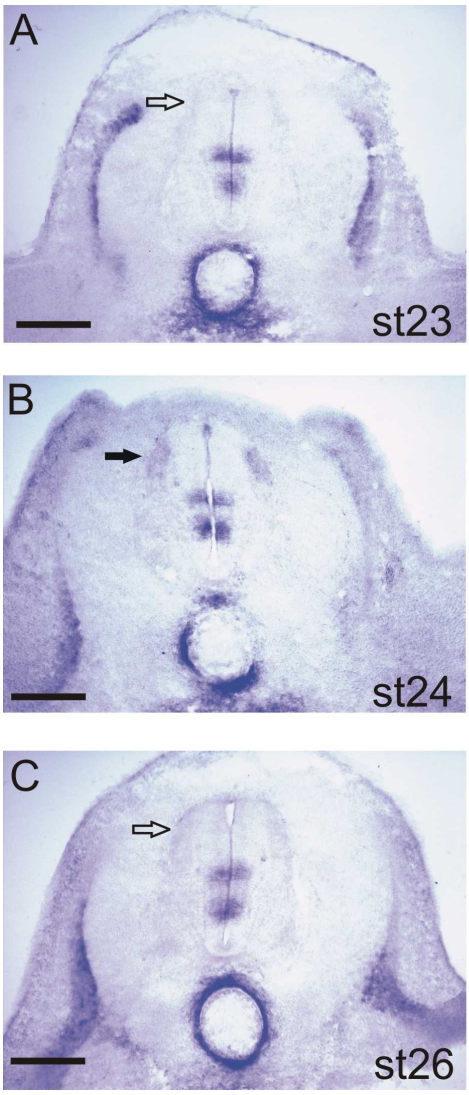


Figure 12 Hip was expressed transiently by commissural neurons during the time when their axons turn into the longitudinal axis (**A-C**). Hip expression was probed by *in situ* hybridization in transverse spinal cord sections at stage 23 (**A**), stage 24 (**B**) and stage 26 (**C**). At stage 23, when commissural axons are crossing the floor plate, Hip was not expressed by commissural neurons (open arrow in **A**). At this stage, its expression in the spinal cord was restricted to two areas of the ventricular zone. At stage 24, when commissural axons have reached the contralateral border of the floor plate and turned into the longitudinal axis, commissural neurons transiently expressed Hip (arrow in **B**). At stage 26, when commissural axons have extended along the longitudinal axis for some distance, Hip was downregulated to very low levels (open arrow in **C**). Bar, 200μm in **A**, 300μm in **B** and **C**. (Adapted from Bourikas et al., 2005)

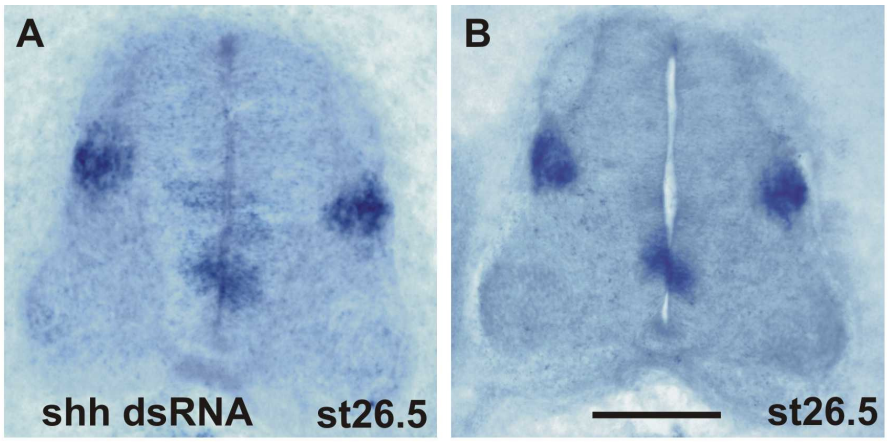


Figure 13 Hip expression in the spinal cord did not change after *SHH* downregulation by *in ovo* RNAi at stage 18-19. To assess whether downregulation of Shh would interfere with *HIP* expression, we compared the *in situ* patterns of stage 26.5 embryos treated with Shh dsRNA (**A**) with an age-matched control (**B**). We chose stage 26.5 because the expression pattern of *HIP* changed significantly at stage 26. A striking upregulation of *HIP* expression in a cell population just ventral of the dorsal root entry zone was seen in both the control embryo (**B**) and the embryo treated with Shh dsRNA (**A**). Note that a slightly younger embryo (**Fig.12C**) does not yet show *HIP* expression in the lateral spinal cord. Bar 300μm. (Adapted from Bourikas et al., 2005)

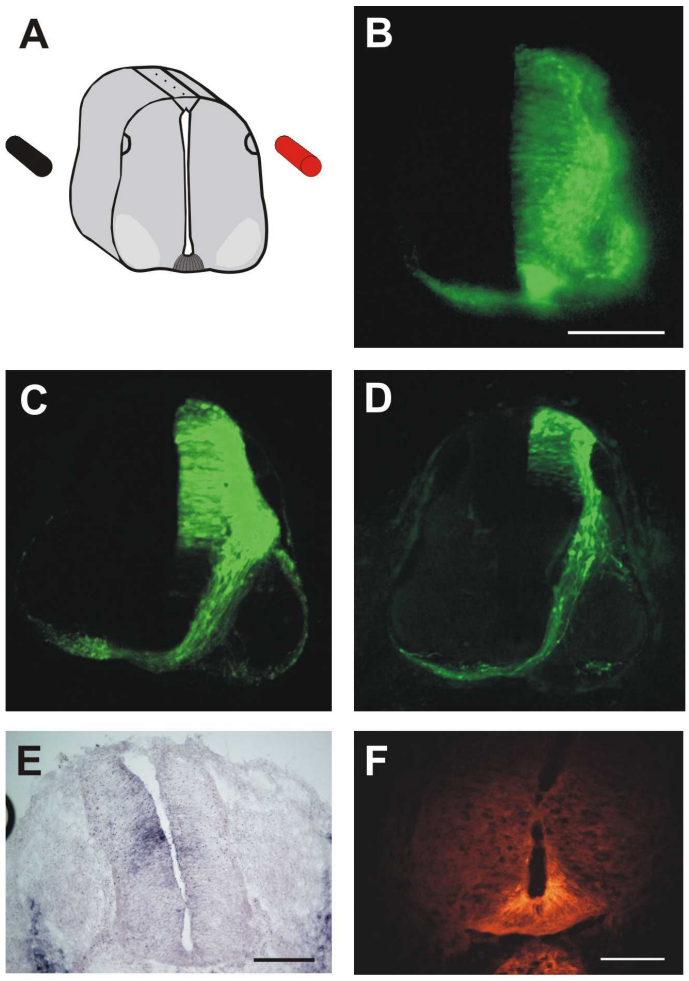


Figure 14 Downregulation of *HIP* in commissural neurons by *in ovo* RNAi. To silence *HIP* by *in ovo* RNAi we injected dsRNA derived from the chicken *HIP* cDNA into the central canal of the spinal cord (see Materials and Methods for details). The electrodes were placed in parallel to the longitudinal axis of the spinal cord at the lumbosacral level (**A**). With this set-up commissural axons could be targeted very efficiently, as shown by the expression of YFP from a co-injected plasmid (**B-D**). Depending on the dorso-ventral position of the electrodes cells all along the dorso-ventral axis (**B**) or only at more dorsal levels (**C,D**) were targeted. The electroporation of *HIP* dsRNA resulted in a decrease of *HIP* mRNA on the electroporated side compared to the control side (**E**). No change of *SHH* expression was seen under these conditions. Bar 300μm in **B-E** and 100μm in **F.** (Adapted from Bourikas et al., 2005)

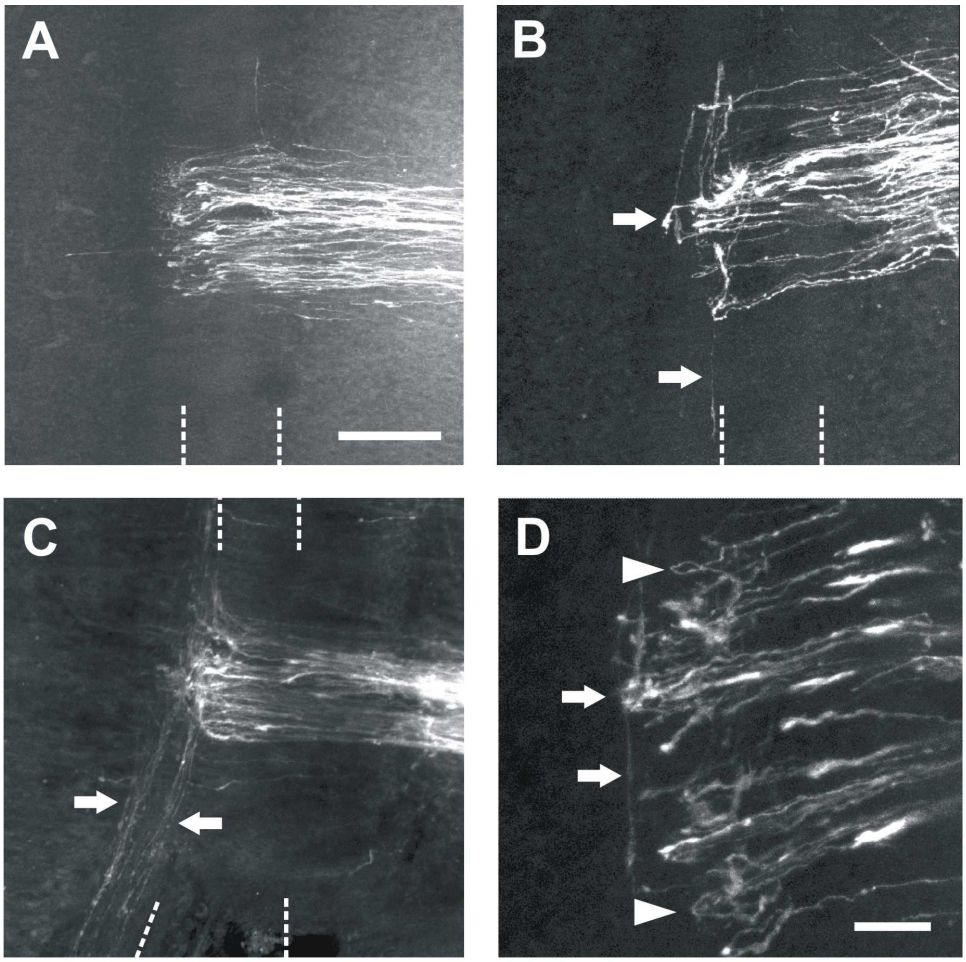


Figure 15 Hip was identified as the receptor that mediated the effect of Shh on postcommissural axons **(A-D)**. Images of open-book preparations from four different embryos in which *HIP* function was abolished by *in ovo* RNAi. Loss of *HIP* function resulted in the same phenotype as loss of *SHH* function (compare to **Fig.5**). Commissural axons did not turn rostrally along the contralateral floor-plate border. Most of them stalled at the floor-plate exit site **(A)** but some of them grew caudally (arrows in **B-D**). As seen after perturbation of *SHH* function , some axons corrected their aberrant initial pathway to grow rostrally by forming loops (arrowheads in **D**). Loop formation and pathway connections were never observed in control-injected embryos (compare **Fig. 4B**) Rostral is toward the top in all panels. The floor plate is indicated by dashed lines in **A-C**. Bar, 100μm in **A-C**, 50μm in **D**. (Adapted from Bourikas et al., 2005)

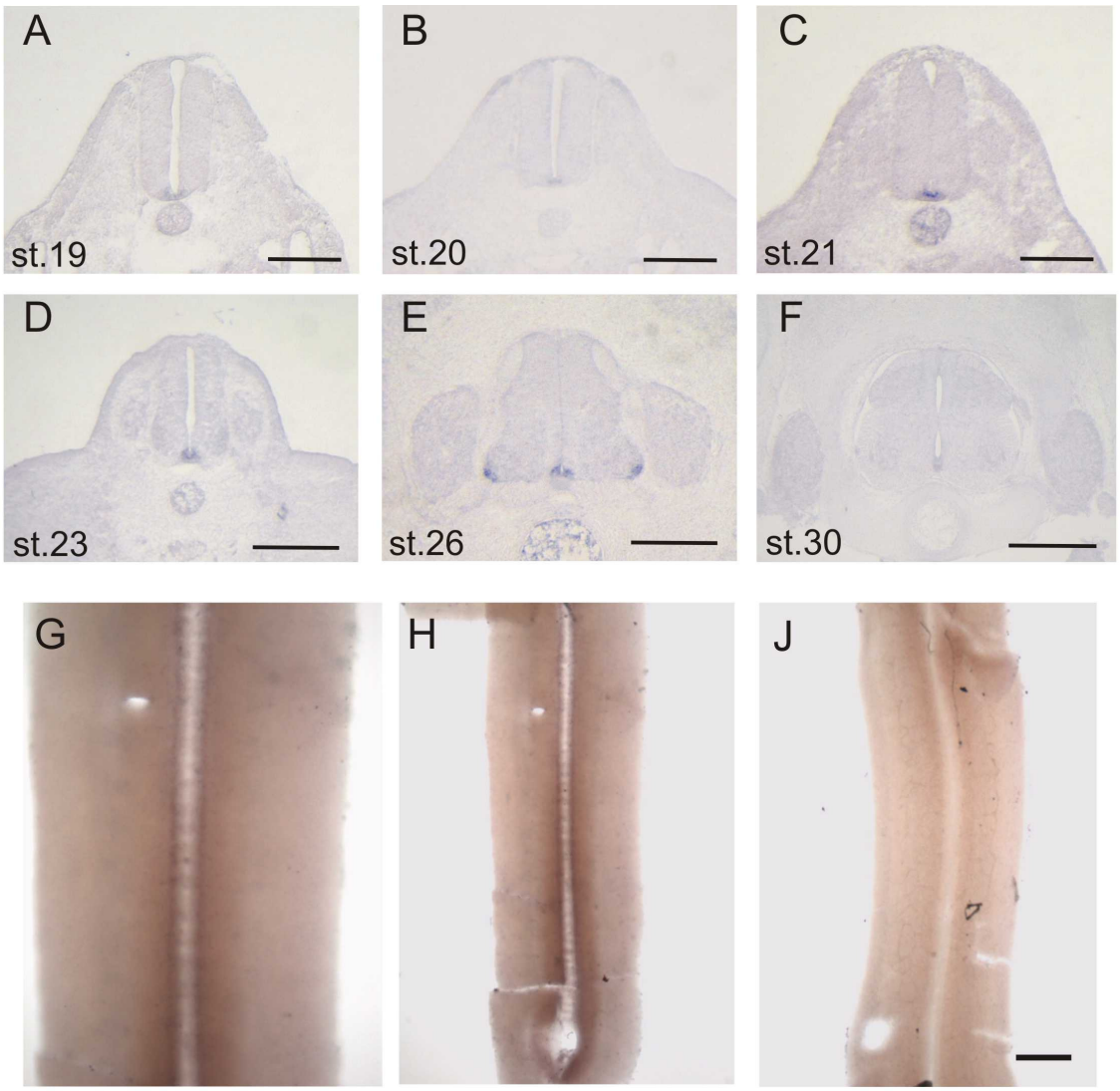


Figure 16 *In situ* hybridization of *SHH* on transverse sections showed expression of *SHH* within floor-plate cells between developmental stages 19-26 **(A-E)** and a reduction in expression levels from this time point onwards. By stage 30 *SHH* is no longer expressed within the spinal cord **(F)**. *In situ* hybridization of *SHH* in an open-book preparation of a stage 25 spinal cord revealed a graded expression with higher levels caudally. High **(G)** and low **(H)** magnification of the same spinal cord is shown. **(J)** *In situ* hybridization in open-book preparation with corresponding sense probe. Bar, 300μm in **A-F** and 500μm in **G-J.** (Adapted from Bourikas et al., 2005)

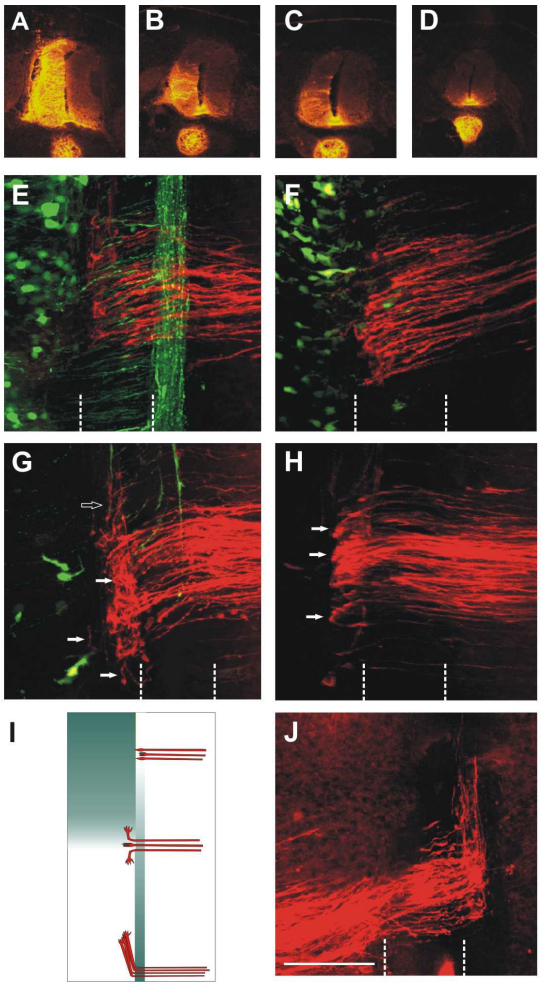


Figure 17 Postcommissural axons avoided high levels of Shh *in vivo* **(A-D).** 5E1 staining of sections taken from different spinal cord levels. Shh was expressed ectopically in a spatially controlled manner in one half of the spinal cord at thoracic **(A)** and upper lumbosacral levels **(B,C).** No ectopic Shh expression was found at caudal lumbosacral levels **(D)**. Postcommissural axons encountering high levels of Shh stalled and did not leave the floor plate **(E,F)**. Axons crossing more caudally, where the ectopic Shh expression was decreasing, showed a lack of rostral bias or even turned caudally (arrows in **G,H**). Very few, if any, axons managed to extend rostrally (open arrow, **G**). Summary of Shh expression and behavior of commissural axons at different spinal cord levels **(I)**. At upper lumbosacral level (top), commissural axons encountered high concentrations of ectopically expressed Shh and did not leave the floor-plate. In the transition zone between ectopic and endogenous Shh expression (middle), commissural axons encountered either no or a reversed gradient and, therefore, responded with either stalling or caudal turns. At caudal lumbosacral levels (bottom), pathfinding was not affected, as axons were exposed only to the endogenous Shh gradient. As a control, we traced commissural axons from the electroporated side of the spinal cord **(J)**. Encountering high Shh before midline crossing, that is, before Hip was expressed, did not change their pathfinding behavior. Floor plate indicated by dashed lines in **E-H** and **J.** Rostral direction is towards the top in **E-J.** Bar 100μm, in **E-H, J** and 300μm in **A-D**. (Adapted from Bourikas et al., 2005)

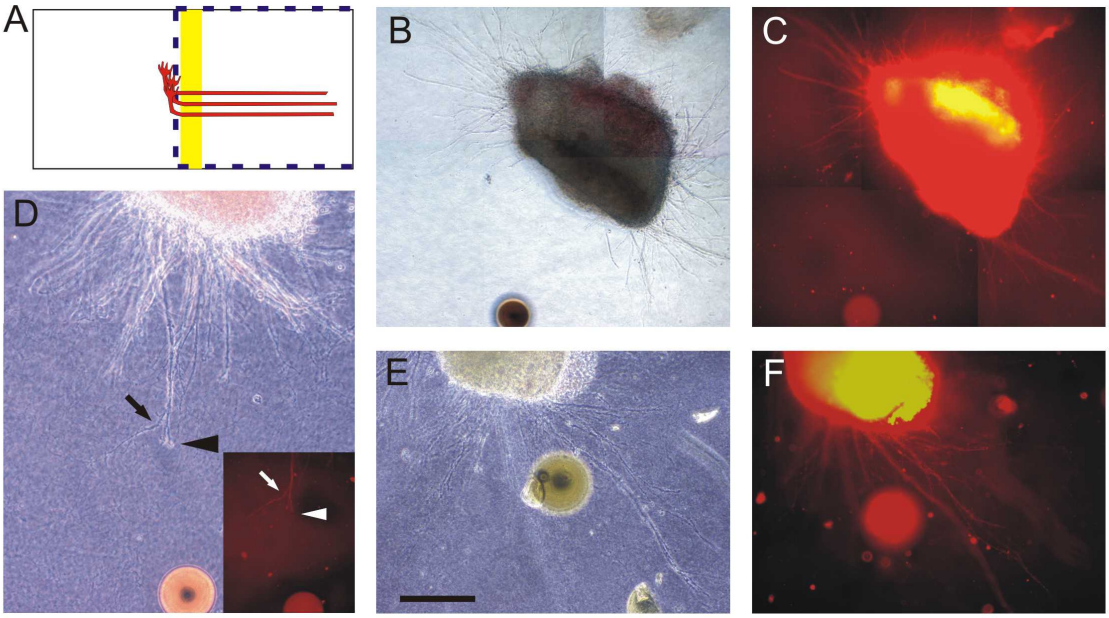


Figure 18 Shh acts as a repellent on postcommissural axons. Postcommissural axons were labeled with DiI in opoen/book preparation of the lumbosacral level of the spinal cord from stage 24 embryos. Explants were cut as shown (blue dashed line) and cultured in collagen gels **(A)**. Beads soaked with either Shh **(B-D)** or in serum albumin **(E,F)** were positioned between 200 and 700um away from the explants. Postcommissural axons did not grow from the edge of the explants facing Shh beads **(B,C)**. Either axons did not leave the explant or they turned away from the bead as soon as they entered the collagen gel.. The bead shown in **D** was positioned more than 900um away from the explant. In that case, axons left the explant also on the side facing the bead, but they either stalled (arrowhead) or turned away from the bead at a distance approcimately 320um. The insert in **D** shows that the axons are, in fact, labeled dorsal postcommissural axons. Control beads coated with serum albumin did not affect the growth of postcommissural axons **(E,F)**. Bar, 200μm in **D-F,** 400μm in **B,C.** (Adapted from Bourikas et al., 2005)

3.2 Identification of guidance cues responsible for the targeting of the central projection of sensory afferents to different laminae within the spinal cord

The sensory neurons in the DRG do not consist of a homogeneous population of cells. A lot of different parameters, such as morphology (Lawson et al., 1984; Rambourg et al., 1983; Plenderleith et al., 1988), conduction velocity, biochemical properties (Boehmer et al., 1989; Carr et al., 1990; Dodd and Jessell, 1985) and function have been used to categorize sensory neurons in different groups. For reasons of simplicity DRG sensory neurons have been classified according to their function into nociceptive, proprioceptive and mechanoreceptive. The functional complexity of DRG sensory neurons is also displayed in the termination of their central afferents in different laminae of the spinal cord. Therefore, it is obvious that different subpopulations of DRG sensory neurons are responsive to different guidance cues present in the spinal cord that direct them to specific sites according to their modality. However, it remains still unknown which guidance cues mediate this precise targeting and moreover which molecular mechanisms are involved in this process.

3.2.1 Segregation of subpopulations of sensory neurons

In order to identify guidance cues that are responsible for the differential termination of the central afferents of DRG sensory neurons we have performed a subtractive hybridization screen in order to retrieve the genes that are differentially expressed by distinct subpopulations. Presumably some of these genes would be acting as guidance cues for the axons of sensory neurons. A prerequisite for a subtractive screen was the segregation of the neuronal subpopulations for the isolation of subpopulation-specific total RNA.

The subpopulations of DRG sensory neurons differ in their dependence on neurotrophins for their survival. It has been shown that nociceptors express *TRKA* receptors and depend on nerve growth factor (NGF) for their survival

(Gorin and Johnson, 1979). Neurotrophin-3 (NT-3) has trophic effects on the *TRKC*$^{+}$ proprioceptive neurons (Maisonpierre et al., 1990) while Brain Derived Neurotrophic Factor (BDNF) has trophic effects ob the *TRKB*$^{+}$ mechanoreceptive neurons (Ibanez et al., 1993). To exploit this difference in a way that would lead to the segregation of neuronal subpopulations of interest we performed limb ablations in developing chicken embryos. By limb bud removal (LBR), the DRG neurons of the lumbar level are destined to undergo apoptosis as the source of neurotrophins, namely the skin and the muscle of the limb, are no longer present (for review, see Oppenheim et al., 1978). Exogenous addition of neurotrophic factors have been shown to be able to block the apoptosis triggered by limb ablation and by doing so to specifically rescue the different subpopulations of DRG sensory neurons (Snider et al., 1994; Caldero et al., 1998).

3.2.2 Limb bud removal triggers increased apoptosis of DRG sensory neurons

The loss of DRG sensory neurons after LBR has not be attributed to altered proliferation, migration, or differentiation (Carr and Simpson, 1978; Oppenheim et al., 1978) but merely to an increase of apoptosis. To reproduce these results we have performed LBR at stage 18 of the developing chicken embryo, a time point during which the neural crest cells have formed the cell aggregate that will give rise to the DRG. Embryos were sacrificed 2, 3 and 4 days after limb removal and processed for TUNEL staining in order to detect the level of apoptosis within the DRG. On transverse sections of limb-ablated embryos (sacrificed two days after LBR) we have observed an increased level of apoptosis within the DRG on the ablated side compared to the contralateral side that was consider a control (**Fig. 19A,B**). Cell counts of the pycnotic cells of the DRGs (n=3) revealed a fourfold increase of apoptosis at the DRG of the LBR side compared to the control (**Fig. 19, Table**).

To assess the increased levels of apoptosis triggered by limb ablation overtime we analyzed embryos that had survived 2, 3 and 4 days after LBR. In this case

we detected apoptotic cells by DAPI staining on transverse sections, as apoptotic cells show a shrivelled and fragmented nucleus (**Fig. 20A,B**). Neurons were stained with anti-NeuN, a neuron-specific antibody. (**Fig. 20A,B**). Cells counts showed a significant increase of apoptosis. The ratio of apoptotic cells to total number of neurons between the LBR side and the control side peaked 3 days after limb ablation and was still high even 4 days after limb ablation (n=3 embryos per treatment; **Fig. 20C**).

Thus, we have demonstrated that limb ablation can trigger increased apoptosis of DRG sensory neurons that lasts at least 4 days after the tissue ablation.

3.2.3 Neurotrophin injections and specific rescue of DRG sensory neuron subpopulations

In previous studies it was shown that different neurotrophic factors can rescue sensory neurons from normal programmed cell death (PCD) in the chick embryo *in vivo* (Oppenheim et al., 1997; Neff et al., 1993; Johnson et al., 1995). Moreover, it was specifically shown that all three neurotrophins (NGF, BDNF, and NT-3) promote the survival of sensory neurons in the L3 DRG of control embryos after daily treatment (5-10µg) from E5 to E9 (Oppenheim et al., 1993). Similarly, the neurotrophins were also the only factors tested that promoted the survival of sensory neurons after LBR (Caldero et al., 1998).

To verify whether we could repeat the results given in the literature we performed unilateral limb ablations at stage 18 (E3) of the developing chicken embryo. The embryos were allowed to develop for 2 days, until stage 24-25, before they were injected with either NGF (10µg/ml), BDNF (20µg/ml) or NT-3 (10µg/ml), three times every 12 hours. Twelve hours after the final injection the embryos were sacrificed at stage 32-33. Immunohistochemistry with the anti-NeuN antibody, that recognizes a panneuronal marker, and subsequent cell counts on transverse sections of limb-ablated and neurotrophin-injected embryos, revealed that all three neurotrophins could rescue DRG sensory neurons from apoptosis. Limb-ablated embryos that did not receive injections of neurotrophins showed a 41%

(292/492) decrease in the number of DRG sensory neurons compared to the number of the sensory neurons from the control side. Injection of BDNF reduced the decrease to 9% (561/612), injection of NT-3 to 7% (543/583) while injection of NGF reduced the decrease in the number of sensory neurons to 16% (604/719). To our surprise an increase in the number of sensory neurons was observed on the contralateral side of the embryo that did not receive any injections of neurotrophins. Presumably some of the exogenously provided neurotrophin reached the contralateral DRG sensory neurons rescuing them from Programmed Cell Death that normally occurs during the development of the nervous system (**Fig. 21**).

The cell counts described above showed that the injection of the three different neurotrophins could rescue, to some extent, DRG sensory neurons from apoptosis triggered by limb ablation. However, they did not provide any evidence about the specificity of the rescue; in other words cell counts do not demonstrate that the injection of a certain neurotrophin led to the specific rescue of a neuronal subpopulation that carried the receptor for the injected neurotrophin. To test the specificity of the rescue we used a quantitative RT-PCR approach. Total RNA extracted from DRGs of limb-ablated and neurotrophin injected embryos was the starting material for the reverse transcription. The resulting cDNAs were subsequently used for PCR using primers specific for amplifying fragments of the *TRKA*, *TRKB* and *TRKC* receptors. These receptors were chosen as they represent the main known markers for nociceptive, mechanoreceptive and proprioceptive sensory neurons, respectively. As an internal control a fragment of actin was also amplified.

To quantify the results of the RT-PCR we have measured the intensity of the bands, normalized these values with the intensity of the bands of actin and blotted them according to the treatment (**Fig. 22A**). The results obtained with this approach showed that the nociceptive (*TRKA*$^{+}$) fibers were the ones least affected after limb removal, most probably they managed to survive by aberrantly innervating neighbouring tissue such as the tail or the skin of the limb that remained at the limb-ablated site. However, injection of NGF in limb-ablated

embryos led to a significant increase of *TRKA* expression, compared to limb-ablated embryos, which suggests a significant increase in the number of nociceptive fibers. Futhermore, NGF had a rescue effect, to some extent, on proprioceptive and mechanoreceptive fibers as well. In contrast, injections of NT-3 in limb-ablated embryos had a specific survival activity on the proprioceptive fibers, but did not change the expression of *TRKB* or *TRKA*. Finally injections of BDNF rescued to a significant degree the number of the mechanoreceptive fibers, compared to embryos that did not receive neurotrophin injection, but also rescued some of the proprioceptive fibers (**Fig. 22B**).

As it is shown with the RT-PCR approach, limb ablations and injections of neurotrophins are not triggering the complete loss of one subpopulation of sensory neurons while rescuing another. However, it seems possible to enrich the DRGs for one subpopulation compared to the others, and thus, this approach was feasible for our purpose of obtaining distinct pools of cells for a subtractive hybridization screen.

3.2.4 Identifying differentially expressed genes in DRGs after limb ablation and neurotrophin injection

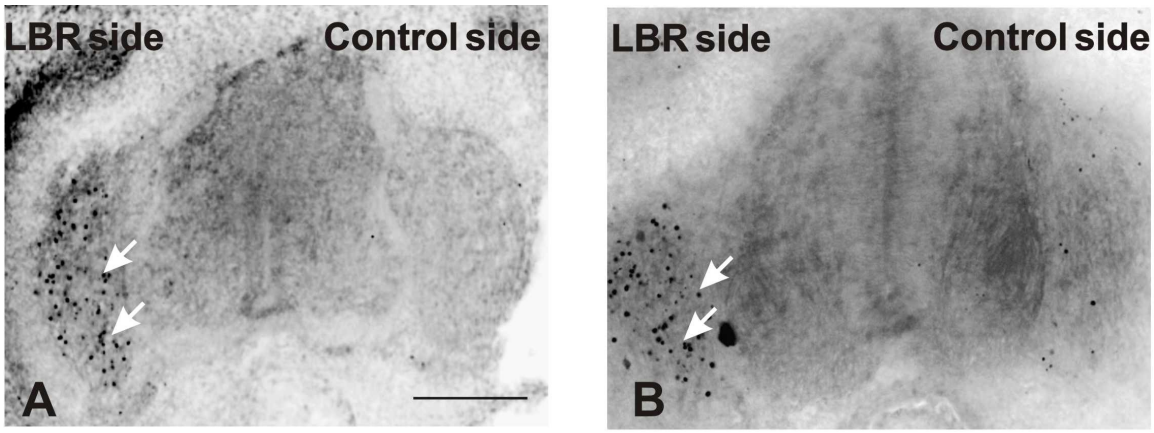
To identify genes that are differentially expressed in DRG sensory neuron subpopulations we used a subtractive hybridization approach, similar with the one used previously for commissural neurons (Bourikas et al., 2005). For that we have constructed 3 different subtractive libraries from DRGs of embryos that were limb ablated and received either NT-3, NGF or BDNF injections. Since injections of neurotrophins led to specific rescue of one of the three DRG subpopulations (nociceptive, mechanoreceptive and proprioceptive) we expected to identify cDNAs expressed in a subpopulation-specific manner.

After forward and reverse hybridization, the differentially expressed cDNAs have been cloned into the pDriver-Vector (Qiagen) that contains two promoters (T7 and SP6) flanking the cloned sequence. The presence of these two promoters

allowed for the production of sense and antisense *in situ* probes as well as dsRNA. Cloned cDNAs were identified by sequencing (**Fig. 23**).

3.2.5 Targeting nucleic acids into the DRG sensory neurons

The candidate guidance cues identified in our subtractive hybridization screen will have to be functionally analyzed. For this *in ovo* RNAi needs to be applied to the developing sensory neurons of the DRG. In contrast to the injection and electroporation at stage 18 that has been shown to efficiently target nucleic acids to neurons in the spinal cord, this approach was inefficient in targeting DRGs. To find the proper developmental stage, the best position for the injection as well as the optimal positioning of the electrodes we used a plasmid expressing yellow fluorescent protein (YFP). After several attempts it was found that our marker plasmid can be taken up by the sensory neurons when injected in the spinal cord and electroporated earlier than stage 17 (**Fig. 24C,D**). To quantify the efficiency of this approach we counted the cells that express YFP and were counterstained with DAPI. Almost 50% of the cells present in the DRG, developing sensory neurons but also Schwann cells, expressed YFP. Moreover, whole-mount staining of an injected embryo with an anti-YFP antibody showed that both DRG sensory neurons as well as motorneurons were transfected with the reporter plasmid (**Fig. 23A,B**). The YFP protein diffused within the axons of the neurons innervating the limb bud.



C

	LBR	Control
Apoptotic Cells	404 ± 20	93 ± 12
Total number of Cells	3020 ± 156	2760 ± 118
Ratio Apoptotic/Total	13.17%	3.30%

Figure 19 TUNEL staining reveals increased apoptosis of DRG sensory neurons after limb bud removal. Embryos were sacrificed 2 days after limb ablation, fixed, cryoprotected and then sectioned. TUNEL staining was performed to detect apoptotic cells. Apoptotic cells were counted in every third section while the total number of cells was estimated with DAPI counterstaining. In the DRG facing the limb ablated side of the embryo an increased number of apoptotic cells was detected (white arrows) compared to the control contralateral side where very few apoptotic cells were present (**A,B**). The average number of apoptotic cells from the lumbar region of three different limb-ablated embryos is shown in **C**. Bar **A,B** 200μm

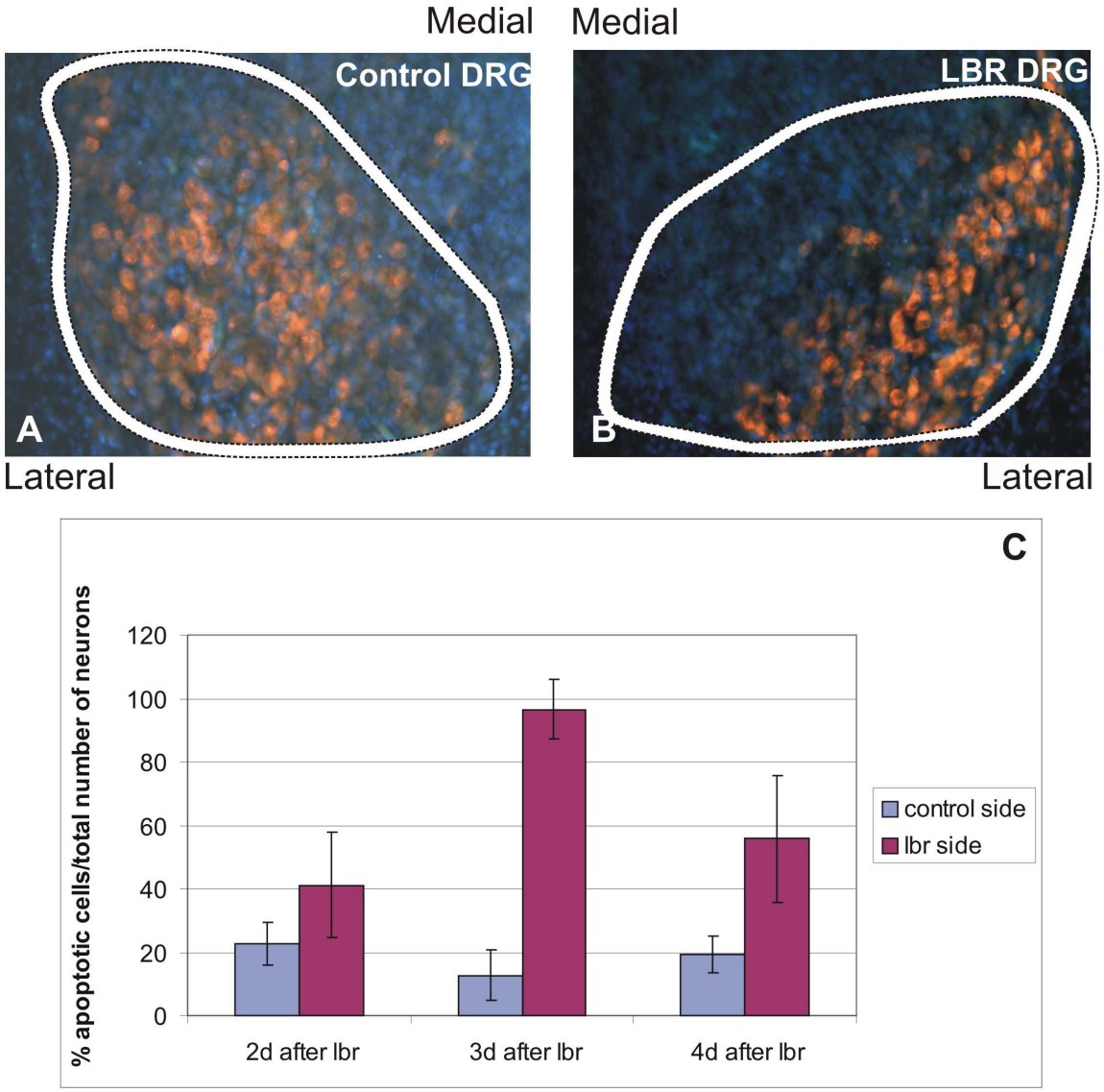
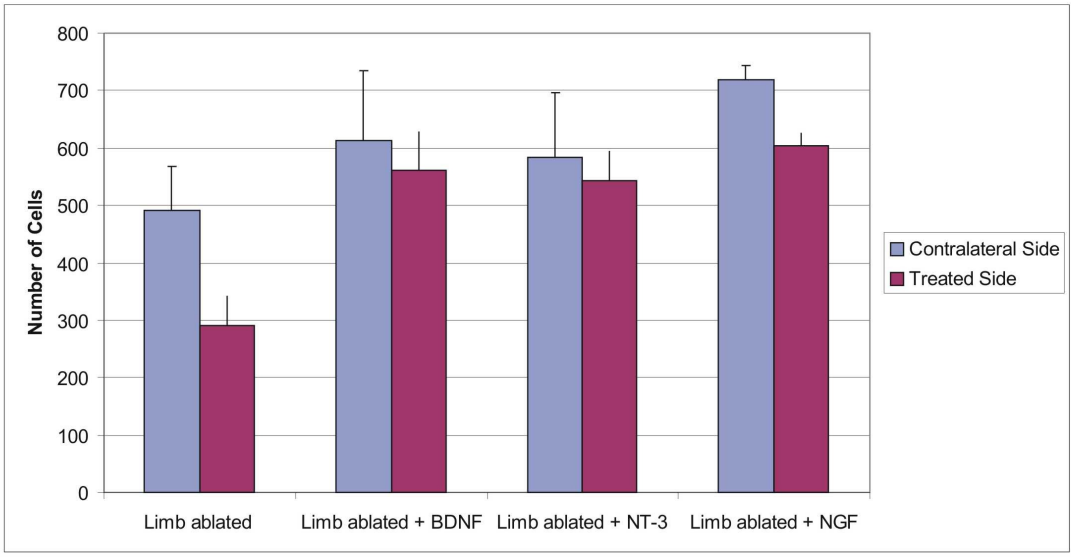


Figure 20 To assess the level of apoptosis after limb ablation, apoptotic cells were identified with DAPI staining, within the DRG. Cells undergoing apoptosis show a shrivelled and fragmented nucleus. Neurons were stained with the anti-NeuN, a neuronal specific antibody (**A,B**). Decreased numbers of neurons were observed in sections of the DRG facing the limb ablated side (**B**) compared to the contralateral control side (**A**). The ratio of apoptotic cells to the total number of DRG sensory neurons was measured in transverse sections of limb ablated embryos over a period of 4 days after limb ablation. Apoptosis of sensory neurons reached a peak 3days after limb ablation and then gradually decreased (**C**). Three embryos were analyzed for each time point. Dotted lines in **A** and **B** outline the DRG.



	Average number of Cells ± SD
Limb ablated	292 ± 51
Control	492 ± 76
Limb ablated + BDNF	561 ± 68
Control	612 ± 123
Limb ablated + NT-3	543 ± 51
Control	583 ± 113
Limb ablated + NGF	604 ± 21
Control	719 ± 23

Figure 21 Cell counts to assess the rescue efficiency of exogenously provided neurotrophins on DRG sensory neurons. Embryos were unilaterally limb ablated and injected three times every 12 hours with neurotophins (NGF, BDNF or NT-3). The embryos were sacrificed at stage 32 and processed with immunohistochemistry with the panneuronal anti-NeuN antibody. Cell counts were performed in every third section and sections from three different embryos per treatment were used. All neurotrophins tested could significantly rescue DRG sensory neurons from apoptosis and the number of sensory neurons in the DRG facing the side of the embryo that received the treatment was similar to that of the number of sensory neurons at the contralateral side.

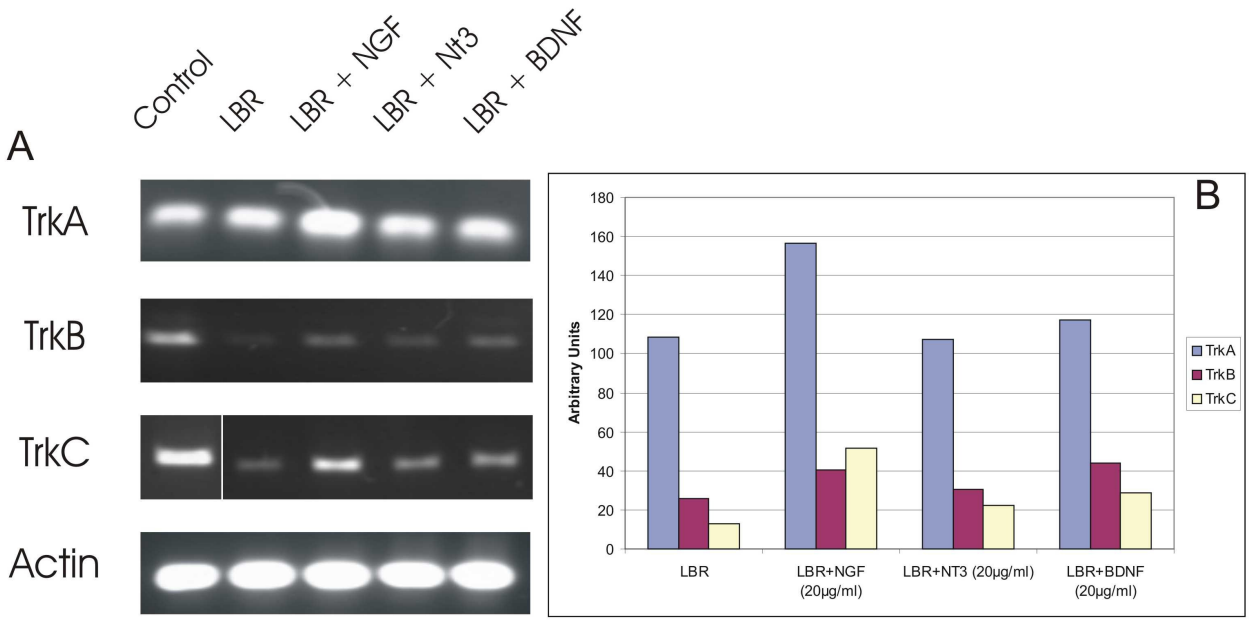


Figure 22 In order to assess the rescue specificity of each neurotrophin (NGF, BDNF, NT-3) on different subpopulation of DRG sensory neurons we have performed an RT-PCR using primers to amplify specifically fragments of *TRKA, TRKB and TRKC*. The total RNA used for this approach was extracted from lumbar DRGs of the limb-ablated side of the embryo that also received the neurotrophin injections. Injections of NGF (20µg/ml) in limb-ablated embryos rescued to a great extent the nociceptive fibes (*TRKA*) and to a lesser extent mechanoreceptive (*TRKB*) and proprioceptive fibers (*TRKC*). Injections of NT-3 (20µg/ml) had a subpopulation-specific rescue effect as it enriched the proprioceptive fibers (*TRKC*) fibers without having any effect on the other sensory neuron subpopulations. Finally, injections of BDNF (20µg/ml) rescued some of the mechanoreceptive but also some proprioceptive fibers.

cDNAs expressed in the DRGs of limb-ablated and NGF-injected embryos

Stathmin	ChEST101f12
ALPHA-TUBULIN (FRAGMENT)	ChEST81g22
NADH-UBIQUINONE OXIDOREDUCTASE	ChEST665a5
Farnesyl pyrophosphate synthetase	ChEST501o22
HEMOGLOBIN ALPHA-A CHAIN.	ChEST973g24
CYTOCHROME C OXIDASE POLYPEPTIDE III	ChEST23n18
60S ribosomal protein	ChEST55i3
Chromatin Assembly Factor 1	ChEST384f16
SR-Related Protein LD2	ChEST889j3
Similar to yeast BET3	ChEST393p5
2810443J12RIK Protein	ChEST691i2
Hemoglobin Epsilon Chain	ChEST24i6
Similar to tubulin beta-2	ChEST35812
RETINITIS PIGMENTOSA GTPASE REGULATOR/FATTY ACID-BINDING PROTEIN, RETINA (R-FABP)	ChEST95h10
Carbonic Anhydrase	ChEST236H23
6230405A16RIK	ChEST402p12
Unknown protein	ChEST1006p13

cDNAs expressed in the DRGs of limb-ablated and BDNF injected-embryos

NON-FUNCTIONAL FOLATE protein	ChEST23k15
CYSTATIN B (STEFIN B)	ChEST142a7
ERYTHROID ANION TRANSPORTER	ChEST668m13
NEUROFILAMENT TRIPLET L PROTEIN	ChEST472k23
EUKARYOTE INITIATION FACTOR 2 BETA	ChEST107k19
UNKNOWN	ChEST563a3
ALR	ChEST673e15
UNKNOWN PROTEIN	ChEST298l14
UNKNOWN PROTEIN	ChEST298l14
SOCS BOX-CONTAINING WD PROTEIN SWIP-1	
KIAA1317 PROTEIN	ChEST1000e7
p38A	ChEST861n9
T-COMPLEX PROTEIN-1, DELTA SUBUNIT	ChEST964g17
HRS PROTEIN (HEPATOCYTE GROWTH FACTOR REGULATED TYROSINE KINASE	ChEST469l17
UNKNOWN PROTEIN	ChEST298l14
TRANSCRIPTION FACTOR HES-5	ChEST295o19

Figure 23 cDNA fragments identified by the subtractive hybridization screen. With this approach cDNAs specifically expressed in *TRKA*$^{+}$ *or* *TRKB*$^{+}$ sensory neurons were subtracted and subsequently cloned in the pDRIVE cloning vector (Qiagen). The selected clones could be used for the preparation of *in situ* probes as well as dsRNA for functional analysis.

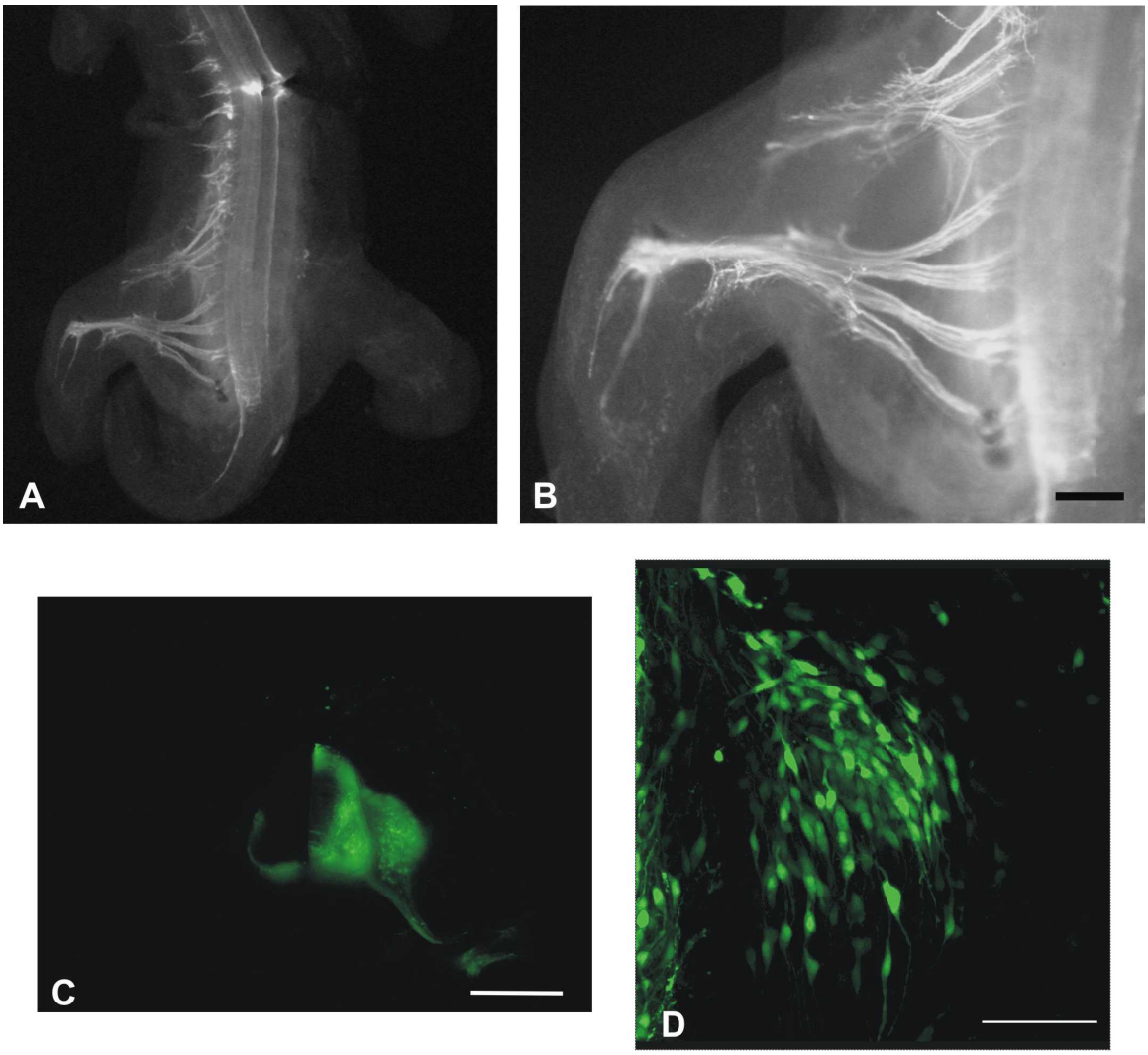


Figure 24 Targeting a plasmid encoding YFP into the DRG. In order to target nucleic acids into the DRGs of the developing chicken embryo, we have injected and electroporated a plasmid encoding YFP into the spinal cord at stage 16-17. The embryos were sacrificed at stage 26 and processed for whole-mount immunohistochemistry with an anti-GFP antibody (**A,B**) or for vibratome sectioning (250um thick sections; **C,D**). Fluorescent microscopy revealed that a considerable number of DRG sensory neurons were expressing YFP (**A,B**) Half of the spinal cord and a great number of DRG sensory neurons of the lumbosacral level expressed YFP (**C**) Expression of YFP in a vibratome section (**D**) DRG sensory neurons were efficiently transfected by injection and electroporation at stage 17. Bar (**A**) 1000um (**B**) 500um, (**C**) 200um, (**D**) 100um (Adapted from Bourikas & Stoeckli, 2003)

4. Discussion

4.1.1 Shh navigates postcommissural axons

Using *in ovo* RNAi, a technique recently developed in our lab to specifically silence candidate genes during commissural axon pathfinding (Pekarik et al., 2003; Stoeckli, 2003) we identified Shh as a guidance cue that directs these axons rostrally along the longitudinal axis of the spinal cord after they have crossed the midline. In contrast to its earlier effects, such as the induction of specific cell populations in the spinal cord (Jessell, 2000) and its chemoattractive effect (Charron et al., 2003) that were mediated by Ptc and Smo, Shh uses a different receptor, Hip, to mediate its effect on postcommissural axons.

A modulatory role of Shh on axon growth during later stages of development was first described for retinal ganglion cell axons (Trousse et al., 2001). Further evidence for a role in axon growth was provided in a turning assay using spinal cord explants, which demonstrated that Shh can attract commissural axons toward the floor plate (Charron et al., 2003). In both cases, the effect of Shh on axon growth was mediated by Ptc and Smo acting as co-receptors (Marigo et al., 1996; Murone et al., 1999).

Notably, Smo and Ptc are no longer expressed by commissural axons once they have grown into the floor-plate area (**Fig.7**). Therefore, it is not surprising that we did not find any effect on commissural axon turning into the longitudinal axis using cyclopamine or *in ovo* RNAi to downregulate *SMO* (**Fig.8**). In search for an alternative receptor for Shh, we turned our attention to Hip (Chuang et al., 1999). Analysis by *in situ* hybridization revealed a highly dynamic temporal and spatial control of *HIP* expression in the developing chicken spinal cord (**Fig.9**). *HIP* is not expressed during the initial phase of commissural axon growth toward and across the floor plate but is transiently upregulated when commissural axons reach the contralateral floor-plate border. After the turn of commissural axons, when they extended along the contralateral floor-plate border, *HIP* was downregulated to barely detectable levels (**Fig. 7c**).

Hip is a type I transmembrane protein but lacks an intracellular domain. Its last 22 amino acids are hydrophobic and have been suggested to form the transmembrane domain (Chuang et al., 1999). Thus, it was unclear how Hip transmitted the Shh signal that resulted in commissural axons` turn into the longitudinal axis. In analogy to Ptc and Smo, Hip could represent a component of a receptor complex that would consist of a Shh-binding unit (Hip) and a signal-transmitting unit that has not yet been identified.

4.1.2 Repulsion: a previously unknown activity of Shh

The graded expression of Shh, with high levels in the caudal-most region of the spinal cord, suggested a repulsive signal (**Fig. 3G-H** and **Fig. 13**). Evidence for a repellent activity of Shh on postcommissural axons was obtained in gain-of-function experiments *in vivo*. We expressed Shh locally in one half of the spinal cord at low thoracic and upper lumbosacral levels. Tracing commissural axons in embryos with a reversed Shh gradient (high levels rostrally and lower levels caudally) resulted in their expected failure to turn rostrally (**Fig. 14**). The repellent activity of Shh on postcommissural axons was confirmed by *in vitro* analysis (**Fig. 15**), where commissural axons were repelled by beads soaked in Shh but not by control beads.

Because Shh is a morphogen (Briscoe et al., 1999) and can act as an attractant for commissural axons (Charron et al., 2003), the temporal and spatial control of gene silencing that is possible with *in ovo* RNAi becomes extremely important. With experiments that interfered with Shh levels before commissural axons had reached the floor plate, we would not have been able to detect the involvement of Shh in commissural axon guidance. Because we blocked *SHH* expression only after stage 18-19, however, either the residual Shh was sufficient to attract commissural axons to the floor plate or, alternatively, *in vivo* the presence of netrin was sufficient to counterbalance the decrease in Shh. Our *in situ* hybridization analysis indicated that the expression of *SHH* dropped transiently between stages 19 and 21 (**Fig.13**). Thus, the downregulation of *SHH* by gene

silencing initiated at stage 18-19 would prevent the effect of Shh on axon guidance but not on axon attraction.

4.1.3 Morphogens act as guidance cues for postcommissural axons

The observation that Wnt4 affects postcommissural axon guidance along the longitudinal axis of the spinal cord in rat and mouse is of great interest in the context of our results (Luyksyutova et al., 2003). Shh has been shown to regulate the expression of secreted frizzled-related proteins (SFRPs; Lee et al., 2000). Sfrps, in turn, are potent inhibitors of the effect of Wnt4 on commissural axon guidance (Luyksyutova et al., 2003). Thus, it is tempting to speculate that Shh, Sfrps, and Wnt4 cooperate in longitudinal axon guidance. High levels of Shh would induce high levels of Sfrps in caudal segments of spinal cord. Therefore, a Wnt4 gradient with the opposite orientation (high rostral to low caudal) would be strengthened by inhibition through Sfrps at more caudal levels. Complementary expression patterns and competitive interactions of Wnt4 and Sfrp2 have been described in chick (Baranski et al., 2000; Ladher et al., 2000) and mouse embryos (Lee et al., 2000). Functional *in vivo* experiments will be required to test for a cooperation of Shh and Wnt4 in postcommissural axon guidance.

4.1.4 Conclusion

Silencing *SHH* by *in ovo* RNAi in a temporally and spatially controlled manner demonstrated the involvement of Shh in guidance of postcommissural axons along the longitudinal axis of the spinal cord. Notably, the morphogenic effects of Shh on spinal cord patterning and the chemoattractive effect of Shh on precommissural axons are mediated by the Ptc-Smo receptor complex, whereas the repulsive effect of Shh on postcommissural axon guidance along the longitudinal axis of the spinal cord is mediated by Hip.

4.2.1 Identification of cDNAs differentially expressed in subpopulations of DRG sensory neurons

To identify candidate guidance cues that mediate the modality-specific targeting of the central processes of DRG neurons into the spinal cord, we have performed a subtractive hybridization screen. A prerequisite for such an approach was the initial enrichment of DRG neuron subpopulations (nociceptive, mechanoreceptive and proprioceptive).

Previous studies have shown that neuronal target fields produce limiting amounts of neurotrophins that promote the survival of the appropriate number of innervating neurons during development (Davies, 1988; Barde, 1989). Experimental manipulation of the availability of neurotrophins can influence the number of neurons that survive, while exogeneously provided neurotrophins prevent naturally occurring cell death in populations of DRG sensory neurons (Buchman and Davies, 1993). Sensory neuron subpopulations express neurotrophin receptors (*TRKA, TRKB, TRKC*) according to their modality; so nociceptive neurons express *TRKA* and depend on NGF for survival, mechanoreceptive fibers express *TRKB* and depend on BDNF for their survival, while proprioceptive afferents express *TRKC* and depend on NT-3 (Snider et al., 1994).

4.2.2 The effect of limb ablation and injection of neurotrophins on DRG sensory neurons

To enrich different subpopulations of sensory neurons within the DRG we have used a combined approach based on limb ablation and exogenous administration of neurotrophins by injections. Limb removal has been shown to result in an increase level of apoptosis of DRG sensory neurons as well as motorneurons due to the removal of the source of neurotrophins (Caldero et al., 1998).

To repeat these observations we have performed unilateral limb ablations in chicken embryos at stage 18. The embryos were sacrificed 2 days later. TUNEL

staining confirmed an increased level of apoptosis of sensory neurons within the DRG on the limb ablated side (**Fig 16**). Cell counts of apoptotic cells revealed a fourfold increase of apoptosis compared to the contralateral, control side. To obtain a better estimation of the number of sensory neurons that undergo apoptosis due to limb ablation we analyzed the time-course of apoptosis. Embryos were unilaterally limb ablated at stage 18 and sacrificed 2 days, 3 days, and 4 days after limb removal. Cell counts with the neuronal marker anti-NeuN revealed that apoptosis due to limb ablation reached a maximum 3 days after limb ablation and declined thereafter (**Fig. 17C**).

Having confirmed that limb ablation was efficient in triggering increased apoptosis in DRG sensory neurons, we also wanted to examine whether exogenously provided neurotrophins could rescue sensory neurons in a subpopulation-dependent manner. Neurotrophins were provided by injections in limb-ablated embryos 2 days after limb removal every 12hours for 1.5 days. Twelve hours after the final injection the embryos were sacrificed at stage 32-33. Cell counts of sensory neurons from limb-ablated, and limb-ablated embryos that received neurotrophin injections have shown that neurotrophins could rescue DRG sensory neurons from apoptosis, triggered by limb removal (**Fig. 18**). Limb ablation could decrease the number of DRG sensory neurons compared to the control side to approximately 40%. BDNF, NGF and NT-3 injection diminished the decreased in sensory neurons numbers to 9%, 7% and 16%, respectively. These results are in agreement with the studies of Caldero et al., where it was shown that injection of neurotrophins could rescue both DRG sensory afferents in a subpopulation-specific manner as well as motorneurons (Caldero et al., 1998). An interesting finding of this study was that neurotrophin injections not only rescued sensory afferents on the limb-ablated side, but also rescued sensory neurons of the contralateral side from normally occurring PCD during development (**Fig. 18**).

Simply counting cells, did not provide any evidence about the specificity of the rescue although it proved that exogenous administration of neurotrophins could rescue DRG sensory neurons from apoptosis after limb ablation. To test whether

each neurotrophin could rescue specifically only one of the different neuronal subpopulations (nociceptive, mechanoreceptive and proprioceptive) we used an semi-quantitative RT-PCR approach for the *TRK* receptors that are reliable markers for these subpopulations (Snider et al., 1994). Injection of NGF (20µg/ml) led to a significant increase of the levels of all three receptors *TRKA*, *TRKB* and *TRKC* suggesting that NGF did not have a specific effect on nociceptive afferents but that it could also rescue mechanoreceptive (*TRKB*$^+$) and proprioceptive afferents (*TRKC*$^+$) to a great extent (**Fig.19**). The unspecific effect of NGF can be partially explained by the fact that within the DRG there are sensory neuron subpopulations which express both *TRKA* and *TRKB* or *TRKA* and *TRKC* receptors (Wright and Snider, 1995). Although the overlap in *TRK* expression had been demonstrated it still remains unclear whether dual *TRK* expression specifies distinct subpopulations. In contrast to NGF, BDNF (20µg/ml) and NT-3 (20µg/ml) rescued specifically mechanoreceptive and proprioceptive afferents, respectively (**Fig.19**).

4.2.3 Identified Clones

In order to identify clones expressed in a sensory neuron-specific manner we have screened cDNAs produced from the mRNA extracted from the lumbar DRGs of embryos that underwent limb ablation and neurotrophin injection, but were not present in limb-ablated embryos. From those embryos that received BDNF and NT-3 injections, we expected to have subpopulation-specific cDNAs, as the rescue of these two neurotrophic factors was specific. Screening cDNAs from limb-ablated embryos that received NGF treatment was expected to identify cDNAs specifically expressing *TRKA* but also *TRKA* together with any of the other two *TRKs*.

Because our approach was based on a significant increase of apoptosis of sensory neurons due to limb ablation in addition to the PCD that takes place during the time period when DRGs were collected, many clones identified in our subtractive screen were found to encode proteins involved in apoptosis.

However, some of the identified clones were have previously been found to be expressed in a subpopulation-specific manner, while some others have also been found to play a role in axon guidance. The most promising clones that might be involved in axon guidance but also in the differentiation process of the different DRG sensory neuron subpopulation are described below.

a. **Nociceptive specific clones**

Stathmin : Stathmin is a ubiquitous cytosolic protein which undergoes extensive phosphorylation in response to a variety of external signals (Sobel, 1991). It is highly abundant in developing neurons and blocking of stathmin with antisense oligonucleotides has been shown to prevent NGF-stimulated differentiation of PC12 cells into sympathetic-like neurons although the expression of several NGF-inducible genes was not affected (Di Paolo et al., 1996). Moreover, it was suggested that stathmin is an essential component of the NGF-induced MAPK signaling pathway and performs a key role during differentiation of developing neurons (Di Paolo et al., 1996)

Retina Fatty Acid Binding Protein (R-FABP) : R-FABP has been found to form a gradient within the developing chicken retina and its expression peaked at the period of cell generation/migration and differentiation (Helle et al., 2002). It is present in cell somata as well as axons and growth cones and its expression has been found to be substratum dependent. Functional studies suggested a possible role of R-FABP in retinal histogenesis and a possibly a regulatory function during topographic map formation (Helle et al., 2002).

LD-2 : Cell surface carbohydrates are though to play important roles in the development and differentiation of mammalian cells. It has been reported that one subpopulation of dorsal root ganglion neurons is specified by the expression of complex globoseries oligosaccharides (Dodd et al., 1984). More complex galactose and fructose-substituted lactoseries structures recognized by MAbs

LD2, KH10, TC6, TD10, LA4 are segregated on subsets of DRG neurons that differ in their expression of substance P, somatostatin and in their terminal termination in the superficial dorsal horn (Dodd and Jessell, 1985). Lactoseries carbohydrate structures such as LD-2 have been implicated in cell-cell interaction.

b. **Mechanoreceptive specific clones**

Chaperonin t-complex protein-1 (TCP-1) : Molecular chaperones assist in the folding of proteins but their role in development is not well understood. Sun et al., has shown that *TCP-1* was expressed in mid-gastrula and later stages of axolotl embryos (Sun et al., 1995). Whole-mount *in situ* hybridization revealed the presence of *TCP-1* transcripts in the brain and spinal cord at the neural stage as well as in the somites at the tailbud stage. The function of TCP-1 in development remains unknown.

Augmenter of Liver Regeneration (ALR) : ALR was initially identified as a growth factor involved in liver regeneration (LaBreque et al., 1975) and it has been implicated in iron homeostasis. A recent study revealed the presence of ALR within the brain. Northern blots showed that *ALR* has a differential expression within the brain with highest mRNA levels within the cerebellum and diencephalon. ALR immunoreactivity was found both in neurons and glial cells throughout the brain (Tury et al., 2005). It is suggested that in the CNS *ALR* might be of importance in heavy metal homeostasis whose dysregulation can induce neurodegenerative disorders (Tury et al., 2005)

Cystatin-B (CSTB) : A mutation in CSTB has been shown to cause progressive myoclonus epilepsy (Pennachio et al., 1996). Mice with a gene deletion of *CSTB* exhibit increased apoptosis of specific neurons but the physiological role of CSTB in brain cells is not fully understood. *CSTB* is also expressed in embryonic

and adult neural crest cells but also in neurons and glial cells (Brännvall et al., 2003). In neural crest cells CSTB is present in the nucleus while in astrocytes can be found additionally in the cytoplasm (Brännvall et al., 2003).

HES-5 : HES-5 is a basic helix-loop-helix transcription factor with a distant homology to *Drosophila* hairy and Enhancer-of-Split proteins (Cartos-Ortega and Knust, 1990). Member of the HES family, such as HES-1 and HES-5 are known Notch effectors, and additional inactivation of HES-3 extensively accelerate cell differentiation and cause a wide range of defects in brain formation (Hatakeyama et al., 2004). In *HES*-deficient embryos, initially formed neuroepithelial cells are not properly maintained, and radial glial cells are prematurely differentiated into neurons and depleted without generation of late born cells. In addition, the forebrain lacks the optic vesicles and the ganglionic eminences (Hatakeyama, 2004). Thus, it seems possible that *HES* genes are essential for generation of brain structures of appropriate size, shape and cell arrangement by controlling the timing of cell differentiation.

SWIP-1 : *SWIP-1* is expressed in somatic mesoderm and developing limb buds as well as in other embryonic structures where Hedgehog signaling has been shown to play a role (Vasiliauskas et al., 1999). SWIP-1 belongs to a novel subfamily of WD proteins but its function remains unknown.

Ran GTPase : Ran is a GTP-binding protein involved in several essential roles for cell viability. However, Ran shows a differentiated expression pattern that is restricted to specific tissues from embryo to adult (Onuma et al., 2000; Koizumi et al., 2001). At early embryonic stages of mouse development there is persistent *RAN* expression in proliferating neural tissue, neural crest-derived dorsal root ganglions and sensory pits (Coutavas et al., 1994).

Apart from the clones identified so far and described above, a great number of clones represent unknown genes when blasted against the Chicken Database

and the NCBI Database. The proteins that originate from these sequences remain unknown.

4.2.4 Conclusion

With the combined approach of limb ablation and neurotrophin injection, we have demonstrated that it is possible to segregate subsets of DRG sensory neurons. Total RNA extracted from lumbar DRGs enriched for one of the three DRG sensory neuron subpopulations were used to perform subtractive hybridization screens in order to identify clones differentially expressed in each of the subpopulations. Several of the clones identified so far have already been identified as markers for subsets of sensory neurons proving the efficiency of our approach. Within the selected clones we expect to identify subpopulation-specific genes that would express proteins responsible for the targeting of the central and peripheral processes of the DRG sensory neurons. Thus, the expression profile of each clone needs to be analyzed with *in situ* hybridization and the function with *in ovo* RNAi. Additionally, it is possible that some of the identified genes/proteins might be used in later studies as subpopulation-specific markers.

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LIST OF PUBLICATIONS

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Ich erkläre, dass ich die Dissertation, Molecular Mechanisms of Axon Guidance, nur mit der darin angegeben Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Zürich eingereicht habe.